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TITLE: Research and Operational Support for the Study of Military Relevant Infectious Diseases of Interest to United States and Royal Thai Government

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Cooperative agreement # DAMD17-01-2-0005 was implemented January 1, 2001 to provide funding support for Royal Thai Army at Armed Forces Research Institute of medical Sciences (AFRIMS) engaged in research activities in collaboration with US Army. Administrative, logistical and scientific personnel required to support the ongoing US Army AFRIMS research efforts, and utilities and maintenance required to support the US Army AFRIMS research effort.

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FOREWORD

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I. INTRODUCTION

A. General

Collaborative studies into infectious diseases of military importance have been conducted at the Armed Forces Research Institute of Medical Sciences (AFRIMS) by both the US Army Medical Component (USAMC) and the Royal Thai Army Medical Component (RTAMC) for 4 decades. Studies leading to develop drugs and vaccines to combat tropical diseases of military relevant importance.

B. Statement of work

Administrative, logistical and scientific personnel required to support the ongoing US Army AFRIMS research efforts, and utilities and maintenance required to support the US Army AFRIMS research effort.

C. US ARMY AFRIMS research efforts at Department of Entomology

Department of Entomology research efforts are the following:

1. Use of GIS to Assess Relative Risk in Different Biotopes within Endemic Scrub Typhus Areas
2. Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines
3. Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

D. US ARMY AFRIMS research efforts at Department of Immunology

Department of Immunology research efforts are the following:

See page 23-32

E. US ARMY AFRIMS research efforts at Department of Enteric Diseases

Department of Enteric Disearses research efforts are the following:

1. Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand

2. Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens

3. Characterization of Enteric Pathogens Isolated from Children in Phnom Penh

4. Characterization of Campylobacter jejuni Isolates

F. US ARMY AFRIMS research efforts at Department of Veterinary Medicine

Department of Veterinary Medicine research efforts are the following:

1. Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta*)/*Plasmodium cynomolgi* Malaria Models
2. Care and Maintenance of Rhesus (*Macaca mulatta*) and *Cynomolgus (Macaca fascicularis)* monkeys and Management of Breeding Colonies
3. Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance/Quality Surveillance Program
4. A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs
5. Characterization and Validation of *Anopheles dirus* Sporozoite-Induced Mouse Malaria Models (ICR mouse/*Plasmodium berghei* and *P. yoelli*) for Screening Exoerythrocytic Antimalarial Drugs

G. US ARMY AFRIMS research efforts at Department of Virology

Department of Virology research efforts are following:

1. The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue
2. A Phase II, Prospective, Randomized, Double Blind, Placebo Controlled Field Efficacy Trial of a Candidate Hapatitis E Vaccine in Nepal, WRAIR #749, HSRRB Log #A-9117.1
3. Prospective Study of Dengue Virus Transmission and Disease in Primary School and Villages in Kamphaeng Phet, Thailand
4. A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naïve Children
5. Training and Workshops
6. Influenza Surveillance in Southeast Asia
7. Sentinel Surveillance for Emerging Diseases Causing Dengue-like or Acute Encephalitis Syndrome in the Philippines (SEDP)

H. US ARMY AFRIMS research efforts at Department of Retrovirology

Department of Retrovirology research efforts are following:

1. Screening and Evaluation of Potential Volunteers for a Preventive HIV-1 Vaccine Trials in Thailand (RN 148, HSRRB)
2. A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp 120 B/E (AIDSVAX® B/E) Boosting in HIV-uinfected Thai Adults (RV144, HSRRB Log No. A-11048, BB-IND 8795)

I. Space and Utilities Required

Funding under the cooperative agreement is also directed by the Principal Investigator to the provision of site maintenance including space and utilities management for both the RTAMC and the USAMC in support of research activities.

II. BODY

A. Department of Entomology, AFRIMS FY05 Research Accomplishments

1. Title of research project: Use of GIS to Assess Relative Risk in Different Biotopes within Endemic Scrub Typhus Areas

a. Investigators:

LTC James W. Jones, PhD;
Kriangkrai Lerdthusenee, PhD
Joe Nigro, MS

b. Objectives:

Geographic information systems (GIS) will be used to explore the spatial relationships of rodent, chigger, and Orientia populations and to assess risk in individual habitats.

c. Methods:

GIS has been used extensively to analyze remotely- sensed data and to predict distributions of plant and animal species. In our study, rodent-hosts and chigger vectors will be collected from residential, rice field, forest edge, and forest habitats in areas highly endemic for scrub typhus. Vegetation distribution within each habitat will be characterized. In each habitat we will establish 2 trapping systems: a transect line of 100 meters and a grid system of 10x10 meters, each consisting of 20 traps. Captured rodent specimens will be identified to species and blood and tissue samples collected. Ectoparasites will be removed from trapped rodents and chigger specimens will be collected for identification and for Orientia assay. Data will be entered into GIS which will be used to define spatial relationships between rodent, vegetation, chigger and Orientia species. The final product will be a predictive tool for scrub typhus risk in biotopes within an endemic scrub typhus area.

d. Results (accomplishments during the period of May 2004 - December 2004):

High resolution satellite imagery has been acquired, processed, and incorporated into ArcGIS in order to analyze rodent and chigger distributions and to identify environmental variables that contribute to suitable ecological conditions for these species. These environmental variables are, in turn, indicative of the presence of scrub typhus. The Quickbird images of the three major study sites (Mae Sad, Mae Chan, and Bangkruai) are allowing for the extraction of geographic, topographic, and environmental information pertaining to these areas. The environmental information being collected through the imagery, as well as, field campaigns consists of vegetation type, density, soil moisture, elevation, and slope. Eventually, this data will be correlated with the data from rodent and chigger collections and passive case detection of

scrub typhus. In this study, rodent-hosts and chigger vectors are being collected from different biotopes associated with endemic scrub typhus areas throughout Thailand. These collection locations include residential areas, rice fields, forest edges, and forest habitats where scrub typhus is endemic. Scrub typhus occurrence is marked by the presence of these fringe habitats where forested areas, rice fields, and villages meet to form an ecotone. Remote sensing and GIS provide the optimum means for identifying these areas and quantifying them. Using higher resolution satellite imagery (ranging from 0.6 meters to 2.5 meters depending on the bands being used) more detailed information will be extracted within each habitat ultimately producing a more statistically accurate risk model. In each habitat, we established 2 trapping systems: a transect line of 100 meters around the house and a grid system of 10x10 meter, each consists of 20 traps (10 small live-traps, 10 medium live-traps). Captured rodent specimens are being identified to species. Blood and tissue samples are collected from animals, kept in cryovials, and stored at -70° C. Each blood and chigger sample is assessed by ELISA and positive samples are assessed by polymerase chain reaction (PCR), with primers specific for Orientia. Samples that are identified as positive for Orientia are examined for further strain identification. All ectoparasites are removed from trapped rodents for identification. Chigger specimens were collected and identified, along with the plant species found in the trap areas. Plant and rodent diversity is governed not only by physical factors found at a site, such as climate, soil type, and elevation but also by local land usage. Classification of local vegetation is being undertaken using state-of-the-art eCognition classification software and will permit correlation of vegetation types with predicted distribution of individual rodent species. We also anticipate that this will allow us to associate vegetation types with animal species regionally. Vegetation density will also be calculated using the normalized difference vegetation index (NDVI).

Maps have been produced; however, the process needs to be evaluated in a new location and currently there is no funding available.

e. Future plans:

Complete unless funding becomes available.

2. Title of research project: Development of a Chigger-Challenge Model for the Evaluation of Candidate Scrub Typhus Vaccines

a. Investigators:

LTC James W. Jones, Ph.D
Dr. Kriangkrai Lerdthusenee, Ph.D
Dr. Benjawan Khuntirat, Ph.D

b. Objectives:

1. Conduct genetic characterization of O. tsutsugamushi infecting 12 colonies of Leptotrombidium chiggers sps. maintained at AFRIMS.

2. Evaluate the ability of each of the 12 chigger colonies to transmit O. tsutsugamushi to laboratory mice. Down-select 4-5 key chigger colonies for further studies. These chigger colonies should be infected with different strains of O. tsutsugamushi and should produce consistent, high infection rates when fed on mice.

3. Focus efforts on building up down-selected chigger colonies to the high levels required for potential vaccine studies.

4. Develop methods for assessing the efficacy of candidate vaccines using the chigger/mouse model. Criteria used to assess efficacy must include quantification of rickettsemia in the mice; however, additional methods (clinical or immunological responses) may also be assessed.

c. Methods:

1. Characterization of Strains/Isolates of Orientia tsutsugamushi: The goal is to characterize the 12 strains of Orientia tsutsugamushi infecting our 12 chigger colonies in order to determine phenotypic and genotypic relationship between different strains

2. Evaluate the efficacy of chigger colonies to transmit O. tsutsugamushi to mice and down-select 4-5 key colonies: Colonies of mites were characterized by PFGE and DNA amplified fingerprinting to identify colony lines with the highest rate of infectivity to laboratory mice. These colonies are being monitored over several generations to ensure a "hot" strain capable of infecting >90% of recipient animals is maintained. In addition, control lines of uninfected chiggers have been monitored. This ensures that the mite colonies that are the best vectors of scrub typhus to be used in the mouse protection model. Additional studies need to be conducted to determine if intrathoracic inoculation of O. tsutsugamushi into uninfected chiggers can be used as a means of developing new colonies of infected chiggers. The ability to rapidly develop colonies of chiggers infected with newly identified strains (to include antibiotic resistant strains) of O. tsutsugamushi allows us to evaluate vaccine candidates against a wide variety of pathogens. Finally, we attempt to characterize mechanisms by which Leptotrombidium deliense and L. chiangraiensis become infected with O. tsutsugamushi, to include analysis of vertical (mite to mite) and horizontal (vertebrate to mite to vertebrate) transmission. The ability to infect mites from infected hosts would allow us to evaluate the ability of potential vaccine candidates to prevent the transmission of O. tsutsugamushi from mites to vertebrates and subsequently back into mites. This ability of a vaccine candidate could have important epidemiological implications.

3. Build-up key chigger colonies to levels sufficient to support vaccine challenge studies: The rearing and maintenance of Leptotrombidium chiggers is a long, slow process. The total life cycle (from egg to egg-laying adult) requires approximately 3 months (this is in contrast to 2-3 weeks for most mosquitoes). Each female chigger will only produce about 1000 eggs over her lifetime. Once a chigger colony is selected for use in vaccine trials, it requires approximately 6 months to build it up to a level required to support the trial.

4. Develop methods for assessing the efficacy of candidate vaccines using the chigger/mouse model. Initial efforts focus on determining the course of rickettsemia over time for the 4-5 strains of O. tsutsugamushi selected for further study and on the development and/or confirmation of diagnostic procedures (PCR, ELISA, etc.) to quantify rickettsemia in challenged mice. We will also evaluate the effect of chigger infection with specific strains of O. tsutsugamushi on potential indicators of immunity, to include lymphocyte transformation, morbidity (as quantified by food consumption, weight gain/loss, activity, etc.), and mortality (time to death following chigger infection).

d. Results:

For the first (single-dose vaccination) group: forty-five (45) 8-week-old ICR mice were vaccinated by a single dose administration of the scrub typhus candidate vaccine (25 µg Kp-r56, 10 µg CpG and 60 µL Montanide per mice).
The second (double-dose vaccination) group: twenty five (25) of 4-week-old ICR mice (25 mice) were vaccinated by a double dose administration of the scrub typhus candidate vaccine (with a 4-week interval for boosting).
The third (triple-dose vaccination) group: twenty five (25) of 4-week-old ICR mice (25 mice) were vaccinated by a triple dose administration of the scrub typhus candidate vaccine (with a 4-week interval for boosting).

Each vaccinated mouse was subcutaneously injected with 120 µL Kp-r56 of the scrub typhus vaccine candidate, 60 µL at each side of dorsum neck areas.

The control groups of non-vaccinated mice consisted of 25, 8-week-old, ICR mice. They were subcutaneously injected with adjuvant (10 µg CpG and 60 µL Montanide per mice). Twenty non-vaccinated mice were challenged by scrub typhus-infected Lc-1 (Karp-like) strain chiggers, while the other 5 are being challenged by non-scrub typhus-infected chiggers.

The orbital bleeding of mice was performed 1-3 days before immunization/vaccination and 1 week before chigger-challenging. Serum samples were collected in order to determine any immune response developed by mice.

Regarding the age of vaccinated mice when they were challenged by scrub typhus-infected Lc-1 (Karp-like-strain) chiggers were as followed: 12-week old mice for the single- and double-boosting groups and 16-week old mice for the triple-boosting group. All vaccinated and control mice were clinically observed for 21 days (3-week-period) post challenging by chiggers.

For the single-dose vaccination of a total of 45 mice, 3 mice survived (7%) after 21-day-observations, in which 2 of them showed no clinical signs of sickness and one mouse become sick and recovered after day 16th. Three mice died at the typical time of death from a scrub typhus infected mite feeding procedure, and 39 mice died (94%) within day 13 to Day 20.

For a double-dose vaccination of a total of 25 mice, 8 mice survives (32%) after 21-day-observations, in which 3 of them demonstrated no clinical signs of sickness and 5 mice became sick and recovered after day 21. No mouse died during the characteristic time of death resulting from scrub typhus infected mite feeding, and 17 mice died (68.00%) between day 13 to Day 20.

For the triple-dose vaccination of a total of 25 mice, 6 mice survived (24 %) after 21-day-observations, in which 3 of them showed no clinical signs of sickness and 3 mouse become sick and recovered after day 21st. No mouse died during the normal period of death by scrub typhus feeding procedure, and 19 mice died (76%) within day 13 to Day 20.

The most important observations were made on those mice which exhibited delayed death observed in all 3 groups. The majority of mice died on day 15 - 16 in all 3 groups 39 out of 78 mice (50%): 22 out of 39 for single-dose vaccinated mice, 7 out of 17 for double-dose vaccinated mice and 10 out of 19 for triple-dose vaccinated mice, respectively, when compared to only 3 mice of the single-dose vaccinated group which died normally on day 9 - 12.

All mice in the control groups of non-vaccinated mice which were challenged by scrub typhus-infected Lc-1 (Karp-like strain) chiggers died on day 9- 12. While another control group of non-vaccinated mice which were challenged by non-scrub typhus-infected chiggers all survived.

The second evaluation of scrub typhus vaccine candidates by assessing protection afforded vaccinated mice by was conducted as follows:

1. Single-dose (immunized) vaccination group: 4-week & 8-week-old ICR mice were immunized by a single dose of a scrub typhus vaccine candidate (25 µg Kp-r56, 10 µg CpG & 60 µL Montanide per mouse).

2. Double-dose vaccination group: 4-week ICR mice were immunized by two doses of scrub typhus vaccine candidate (25 µg Kp-r56, 10 µg CpG & 60 µL Montanide per mouse);

Each immunized mouse was subcutaneously injected at each side of the dorsum neck areas. They were challenged (fed on) by scrub typhus-infected Lc-1 (Karp-like-strain) chiggers.

The control groups consisted of 4-week-old non-vaccinated ICR mice which were being subcutaneously injected with adjuvant (10 µg CpG and 60 µL Montanide per mice). One group of 10 non-vaccinated mice was challenged with scrub typhus-infected Lc-1 strain chiggers, while another group of 10 non-vaccinated mice was challenged with uninfected chiggers.

Orbital bleeding of mice was performed 1-3 days before immunization/vaccination and 1 week before chigger-challenges began. Serum samples were collected in order to determine

immune response. Experimental mice were weighed daily and their body weights recorded. Mice were observed daily for clinical manifestations for 21 days (3-week-period) post challenging by chiggers.

Result of 2nd (Scrub Typhus Vaccine Kp r56) Vaccine Trial--- Challenge with Orientia tsutsugamushi-infected Leptotrombidium chiangraiensis (Lc-1) chiggers

1. Immunized by a single-dose of Kp r56 vaccine: Results from our 2nd (Scrub Typhus) Vaccine Trial on 4-week-old mice which were immunized with a single-dose of scrub typhus Kp r56 vaccine showed 3 of 10 mice survived for the period of our clinical observation (21 days period). The survival rate in our 2nd Vaccine Trial is higher than the results of the 4-week old mice challenged with a single-dose of Kp r56 vaccine in the 1st (Scrub Typhus) Vaccine Trial which only 3 of 45 mice survived and also higher than the results of 8-week-old mice inoculated with a single-dose of Kp r56 vaccine in which only 1 of 10 mice survived challenge.

Of the three of ten 4-week-old surviving mice, 2 mice became ill on day 11 and another mouse on day 12. Two mice recovered on day 18 and one mouse on day 20. The single surviving 8-week-old mouse became ill on day 9 and recovered on day 12.

Survival time of the mice that became sick and eventually died was increased over the challenged non-vaccinated group. The normal time for mice to die from challenge normally falls from day 10 to day.

1st Vaccine Trial: 13 out of 42 four-week-old mice with a single-dose of Kp r56 or 30.95% died between days 10 - 13.
2nd Vaccine Trial: 2 out of 7 four-week-old mice with a single-dose of Kp r56 or 28.57% died between days 10 - 13.
2nd Vaccine Trial: 3 out of 9 eight-week-old mice with a single-dose of Kp r56 or 33.34% died between days 10 - 13.

Mice appeared to survive longer in our 2nd vs. the 1st Vaccine Trial:

1st Vaccine Trial: 11 out of 42 (5 on day 15 + 3 on day 16t+ 3 on day 17) four-week-old mice with a single-dose of Kp r56 or 26.18% died on day 15 - day 17,
2nd Vaccine Trial: 3 out of 7 (2/7 on day 15 + 0/7 on day 16 + 1/7 on day 17) four-week-old mice with a single-dose of Kp r56 or 42.85% died on day 15 - day 17,
3rd Vaccine Trial: 4 out of 9 (2/9 on day 15 + 0/9 on day 16 + 2/9 on day 17) eight-week-old mice with a single-dose of Kp r56 or 44.44% died on day 15 - day 17, also in this group one mouse died on day 19.

2. Immunized by a double-dose of Kp r56 vaccine:

Results from challenges during our 2nd (scrub typhus) Vaccine Trial on 4-week-

old mice immunized with a double-dose of scrub typhus Kp r56 vaccine showed 3/10 mice (30%) survived through out the course of our clinical observations (21 days period). The survival rate of 30% in our 2nd Vaccine Trial is similar to the results of the 4-week old mice challenged with a single-dose of Kp r56 vaccine during the 1st (scrub typhus) Vaccine Trial (32%). Of those 3 surviving mice observed in our 2nd Vaccine Trial, all 3 mice exhibited a delayed onset of illness by becoming ill on day 12, one mouse recovered on day 14, while the other 2 mice recovered on day 20. While the other 7 mice in this 2nd Vaccine Trial group died, these mice survived longer than normal from challenge.

When compared the results obtained from the 1st & 2nd scrub typhus vaccine trials of the double dose immunization, it appears that more mice in the 2nd group exhibited delayed onset of illness than the 1st group as well as more mice survived longer.

Based upon our preliminary results on the scrub typhus vaccine trials on either the single-dose and/or double-dose immunized mice, which revealed similar protection in both groups. Results are still inconclusive as to which regime would provide a better protection.

e. **Future Plans:**

Continue to conduct mouse-challenge evaluation of candidate vaccines in FY06.

3. Title of research project: Production of *Plasmodium vivax* Sporozoites to Support a Human Challenge Model

a. Investigators:

LTC James W. Jones, PhD.
Dr. Jetsumon Prachumsri, Ph.D

b. Objectives:

Establish procedures to produce qualified and reliable sources of Plasmodium vivax parasites to support a human challenge model.

c. Methods:

1. Validation of basic system to provide sporozoite-infected mosquitoes in support of STEP/STO requirements. This system will be based on our current ability to feed mosquitoes directly on P. vivax-infected patients reporting to local malaria clinics. In brief, adult patients report to local malaria districts when they think they may have malaria. Ministry of Public Health (MOPH) personnel make thick blood smears and check the patients for malaria. As part of an approved Human Use Protocol, Department of Entomology personnel allow to draw 20 ml of patient blood to feed mosquitoes using membrane feeding technique. These studies are conducted

weekly at Mae Kasa and Mae Sod malaria clinics. Aliquote of each blood sample will be spotted on filter paper and smeared on glass slide. Confirmation of parasite species will be accomplished by PCR of filtered blood and microscopic examination of blood smears. Infected mosquitoes are returned to AFRIMS and maintained in the AFRIMS insectary. Five to 10% of mosquitoes from each mosquito feed are checked for the presence or absence of oocysts approximately 7-10 days after infection. These mosquitoes are thereafter available for use in malaria sporozoite challenge studies.

2. Refined Sporozoite Challenge System (this study was postponed from 2002 to 2003 due to delay in approval of protocol for human challenge study). In 2004 we will continue refining the system in order to reduce the variability in the mosquito infections (critical for ensuring consistent challenges) and to eliminate the risk of concomitant mosquito infections. The goal will be to develop a system that will i) consistently provide mosquito infection rates with >60% of blood-fed mosquitoes having +3/4 (>100 sporozoites) salivary gland infections, and ii) provide P. vivax-infected mosquitoes that do not harbor concomitant pathogens. Consistency in the challenge is a critical component of any vaccine trial. Plasmodium vivax-infected patients reporting to local malaria clinics will serve as the starting point for development of the "refined system". Mosquitoes will be fed on venous blood provided to them in an artificial membrane feeding system. A series of carefully controlled experiments will be conducted using the membrane feeding system. We hypothesize that pooling blood from infected patients will reduce inherent variability in mosquito infections. In addition, pooling blood from several patients may offer the added benefit by increasing the genetic diversity of the sporozoite challenge, and thus may more truly evaluate the efficacy of any candidate vaccine. Replacement of Patient Sera with Commercial Sera: Blood will be collected from patients, and packed red blood cells separated from the sera and subsequently reconstituted with commercial sera. The reconstituted blood will be fed to mosquitoes in a membrane feeding system and mosquito infections quantified. This method has the advantage of removing anti-malaria antibody that may affect gametocyte infectivity (2) and replaces patient sera that is potentially infected with concomitant acellular pathogens with commercial sera that is known pathogen-free.

3. Parasite Characterization: In the absence of an in vitro culture system, it will be necessary to feed mosquitoes on a P. vivax-infected volunteer or the blood from a volunteer. Since it will be impossible to ensure that mosquitoes are infected with a single P. vivax clone (as is currently done with P. falciparum), it is critical that we develop a method of characterizing the parasites (i.e., genetic diversity of the parasites, resistance to antimalarial drugs, etc.). Once mosquitoes are infected, parasites from the infectious blood meal will be characterized by PCR using polymorphic gene targets, such as the nonapeptide repeat region of the circumsporozoite protein (PvCSP), and the region between interspecies conserved blocks 5 and 6 of the merozoite surface protein (PvMSP1)(3,4,5).

4. We will continue our effort on establishing of continuous culture of P. vivax and verifying our technique for short term culture to provide infective gametocyte for mosquito feeding. Use of short term culture technique to produce gametocyte and study gametocyte infectivity. In this portion of the study we propose to evaluate the ability of produce infective

gametocyte by in vitro culture of cryopreserved blood to infect mosquitoes. Effort will focus on establishment of cryopreservation techniques that will maximize parasite viability. Development of procedures to culture infective gametocytes would allow for production of sporozoites from P. vivax specimens obtained from throughout the world. These sporozoites could then be used in a variety of experimental models (i.e., we could use sporozoites obtained from East Timor in our hepatoma cell model to evaluate resistance of Exo-erythrocytic stage parasites to primaquine and tafenoquine). We have used reticulocyte enriched from cord blood as a host for the parasites in our system. One third of P. vivax culture maintained in McCoy'5A medium with 25% AB serum and human reticulocytes can survived up to 37 days in our system. Induction of gametocyto-genesis using McCoy'5A medium with 25% AB serum containing hypoxanthine for the culture of P. vivax will done on at least 100 cases of blood in 2004. The gametocytes from culture will be fed to mosquitoes to verify their infectivity and compare with gametocyte infectivity of the same isolates freshly fed to the mosquitoes without being cryopreserved and cultured.

d. Results (accomplishments during the period of January 2004 - December 2004):

P. vivax culture

1. Isolated CD133+ cells from umbilical cord blood, with 95% of purity, were cultured for 8 days in supplemented medium. These cells were phenotyped by flow cytometry and showed CD34+, CD71+. The cells were expanded to 50-100 folds after one week in the same medium and expanded to 200-500 folds after 3 weeks. The one week old cells have been preserved in liquid nitrogen for futher experiments.

Starting from 9 days old the cells [nucleated + enucleated red cells] were used continuously to culture P. vivax (prepared from patient blood)in McCoy's medium with 25%AB serum. Cells from the P.vivax culture were taken 3 times a week for detection of the parasite by

A: Giemsa staining
B: Immunofluorescence assay
C: RT-PCR

Characterization of target red cells for P.vivax parasite is underway. The culture conditions are under optimization to enhance parasite invasion. Method with specificity and sensitivity is needed for screening of the P.vivax culture.

2. Genotyping analysis of Plasmodium vivax isolates from Mae Sot:
A. Total cases collected from Oct 04- 15 Jul 05 = 106 cases. There were 81 cases of VK210, 5 cases of VK247 and 20 cases of mixed VK210-VK247.

Analysis of MSP3-alpha

1. Analysis of MSP3-alpha by PCR-RFLP was done from 6 samples (1 sample = VK247, 5 samples = VK210) showed 3 patterns already. Sequencing of this gene is on the way.

Evaluation of gametocyte infectivity of 100 isolates frozen with different cryopreservation and protocols show no infectivity of gametocyte after being frozen and thawed. Short term culture of frozen samples has been started.

2. In vitro culture of *P. vivax* using RBC differentiated from stem culture demonstrated that the parasites could multiply and maintain in the culture up to 85 days

e. Future plans:

Continue in FY06.

B. Department of Immunology AFRIMS FY05 Research Accomplishments

1. Title of research project:

Number	Projects	Status
1	MRDD Phase III (687-2001)	In life completed 2002; report writing completed; FDA-IND in process
2	MRDD Phase IIIb – Venous vs. Fingerstick	In life completed 2003; data analysis complete
3	Comparison of PCR adjusted HRP immunoassay vs conventional microscopy for Pf diagnosis	Completed; manuscript submitted
4	Development of new cost effective HRP2 ELISA for Pf malaria drug sensitivity testing	Manuscript submitted
5	Human Malaria Vivax Challenge	Protocol in development
6	Rhesus Pf AMA-1 Combinations Vaccine	Study complete; publication in progress
7	Immunologic studies of SIV/p27 adenovirus as a model system for future malaria antigen studies	Study completed; Data analysis in progress
8	PvDBP Polymorphisms	Study completed; publication in progress
9	Tafenoquine Cure/Radical Cure Dose Ranging NIH	In life completed Jan 2005; manuscript preparation in progress
10	Phase II Assessment of Azithro-Quinine Combos	In life completed; results reported at ASTMH; publication in progress
11	Azithro-Quinine and Azithro Artesunate in Pf Rx	Study completed; publication in progress

12	AS Bioequivalence study (comparing WRAIR IND formulation vs. Guilin non GPO product)	Protocol prepared; scientific approval obtained; study on hold
13	Bioassay/HPLC/LC-MS Validation - FDA	AS complete; LC-MS equipment validated; Method Validation protocol in progress
14	Artesunate Phase II Protocol Development	In progress; in life anticipated late 2006
15	Fever Surveillance in Sangklaburi	Results reported to local health authorities; manuscript published
16	Cambodia Malaria Prevalence Study	In life and slide reading completed; initial data analysis completed; publication in progress
17	Leptospirosis in Sangkhlaburi	Manuscript published; PCR development in progress
18	Molecular Assessment of Nepal Malaria Isolates	In life and data analysis completed; manuscript prep in progress
19	Bangladesh In Vitro Pf Resistant assessment	Protocol approved; Second of 5 year project completed
20	Cambodia In Vitro Pf Resistant assessment	Protocol development and field site assessments
22	Thailand In Vitro Pf Resistant assessment	ongoing; initial data analysis complete; publication in progress
23	PF MSP-1 Genotyping – assay development	in progress;
24	Vivax Genotyping - PV Mahidol	Data analysis
25	Gametocyte Production for Entomology	In Progress
26	Set up and Maintenance of Regulated LC-MS Laboratory	In Progress
27	Pharmacodynamic/kinetic of lead antimalarial drugs in rhesus malaria model	In Progress
28	Safety and Immunogenicity of *Plasmodium vivax* circumsporozoite Vaccine in Rhesus Monkeys	Protocol submitted
29	Development of Real Time PCR Assay for Diagnosis of *Plasmodium falciparum and vivax*	In Progress
30	Development of PCR Assay for Diagnosis of Leptospirosis	In Progress
31	Surrogate Markers of Malaria	Data generated; report and publication in progress

32	Pre-clinical Safety and Immunogenicity Primate Studies to Support INDS	Data generated; publication in progress
33	Evaluation and Testing of *P. falciparum* Parasite Antigens for Use in Active Immunizations Against Malaria	Data generated; publication in progress

a. Investigators:

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Dr. Sathit Pichyangkul, Ph.D.
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b. Objectives:

1. To protect, project and sustain the military soldier against disease threats produced by the 2 major species of malaria, ***Plasmodium falciparum*** (Pf) and ***Plasmodium vivax*** (Pv). To support this mission through the evaluation of new or improved vaccines, prophylactic and therapeutic drugs, rapid diagnostic kits, and the maintenance of a center for excellence focused on the basic biology and epidemiology of malaria.

2. To assess emerging febrile diseases along high-risk regions in Thailand and throughout SE Asia

c. Methods:

The Department of Immunology and Medicine has applied as many kinds of classical and state-of-the-art technologies as possible to the above multi-faceted research. Clinical research included mobile epidemiology team able to work in adverse conditions where malaria is present, including field sample collection and processing screening, reference microscopy, assessment of rapid diagnostics for various tropical infectious diseases, and a staff well-versed in conduct of clinical trails to GCP and ICH standards. The animal research teams are all trained in laboratory animal research and regulations, current AALAAC requirements, and laboratory animal test and observation methods. State-of-the art methodologies are available for the study of vaccine and drugs to include advanced molecular biology methods such as sequencing, SNP analysis, and real-time PCR. Cellular immunology techniques are available which include flow cytometry and sorting technologies, ELISPOT, and molecular methods. Pharmacology assays include HPLC, LC-MS, a unique malaria bioassay to measure the in vivo antimalarial bioactivity of potential

new antimalarial medications, sustained malaria cell culture and radioisotopic uptake, and antibody based methods for measuring in vitro drug sensitivity patterns of malaria strains against standard malaria drugs.

d. Results (accomplishments during the period of January 2005 - December 2005):

1. Malaria Vaccines STEP F/STO AF/STO A1

Completed a study on "Safety and immunogenicity of *Plasmodium falciparum* LSA1/AS01B alone or concurrent administration with RTS,S/AS01B at opposite site" supported by MIDRP. The specific aims were a) to evaluate safety and immunogenicity of LSA1 formulated with the proprietary adjuvant AS01B and b) investigate the interference of safety and immunogenicity profiles elicited by the simultaneous vaccination of LSA1/AS01B and RTS,S/AS01B in opposite legs compared to those induced by the corresponding individual vaccine alone. Rhesus monkeys received 50μg in 0.5 ml of adjuvant of each vaccine at weeks 0, 4, and 8. The results showed that LSA1/AS01B was safe and immunogenic. Specifically, the vaccine induced high magnitude of T cell responses as monitored by intracellular cytokine staining (IFN-g, IL-2). The magnitude of LSA1-specific $CD4^+$ T cell responses peaked after the second dose of vaccine. Surprisingly, the third dose of vaccine led to a significant reduction of $CD4^+$ T cell responses (P=0.017). Animals receiving RTS,S/AS01B showed moderate $CD4^+$ T cell responses to CSP and HBsAg. Similar to LSA1/AS01B, RTS,S-induced $CD4^+$ T cell responses peaked after the second dose of the vaccine. Insignificant LSA1- and CSP-specific $CD8^+$ T cell responses were detected, whereas, significant $CD8^+$ T cell responses to HBsAg were observed. Study on the duration of antigen-specific $CD4^+$ T cell responses indicated that the responses persisted for at least 3 months after the last dose of vaccine. Simultaneous administration of LSA1/AS01B and RTS,S/AS01B each in opposite legs did not alter safety or immunogenicity profiles. No statistically significant differences were observed in the kinetic, magnitude, or duration of antigen-specific $CD4^+$ T cell responses in animals receiving either single or double vaccinations. The antigen-specific antibody responses are currently under investigation. The results from this study will facilitate the design of multi-antigen malaria vaccines. Manuscript is being prepared.

Study on "Prime boost vaccination of Simian Immunodeficiency virus (SIV) Gag in rhesus monkeys" supported by GSK was completed. The main objective of this study was to evaluate different prime-boost approaches for the induction of optimal $CD4^+$ and $CD8^+$ T cell responses. Since the CTL epitope that binds to MHC class I Mamu A01 is known, SIV gag p27 was used as a model antigen. The analysis of $CD4^+$ and $CD8^+$ T cell responses using intracellular cytokine staining and tetramer staining were completed. The antibody-responses are currently under investigation by GSK. Over all, the results demonstrated that specific prime-boost regimen induced a balance of $CD4^+$ and $CD8^+$ T cell responses. However, full data details have to be kept confidential at this time until GSK completes antibody response data.

Continued efforts to develop a new antigen delivery system by targeting dendritic cells (supported by MRMC's Competitive Commander's Grant program). Toll-like receptors

(TLRs) are screening receptors of the innate immune system, recognizing a broad variety of pathogens. Due to their potent role in activation of dendritic cells, ligands for TLRs are attractive candidates for adjuvant formulations. Based on their close relationship to humans, non-human primates, such as rhesus macaque monkeys, have proven to be valuable animal models for testing vaccines and immunization strategies. However, so far, it is not known if a similar set of TLRs is present on dendritic cells from human and rhesus. Therefore, we firstly analyzed TLR expression and function on rhesus dendritic cells. We used an optimized protocol developed by our own group for the generation of rhesus monocyte-derived dendritic cells and analyzed expression of TLRs by PCR. In addition, we also evaluated the ability of several TLR ligands to stimulate rhesus dendritic cell maturation and cytokine production using 4-color flow cytometry. Our results showed that rhesus dendritic cells responded to TLR2, TLR3, TLR4 and TLR5 ligands by up-regulation of co-stimulatory molecule, but not to ligands TLR7, TLR8, and TLR9. In comparison, human monocyte-derived dendritic cells were shown to be activated by the same TLR ligands. Unlike human monocyte-derived dendritic cells, rhesus dendritic cells produced very low amount of IL-12 p70. Interestingly, high levels of IFN-a production were induced by Poly I:C (TLR 3 ligand). It is known that murine dendritic cell subsets substantially differ from their human counterpart. We demonstrated that non-human primate's dendritic cells share functionality of TLRs with human dendritic cells. Our results indicate that rhesus monkeys will be useful for testing novel antigen delivery systems containing TLR ligands. We are now testing the ability of TLR ligands for the generation of antigen-specific CD8+ T cells *in vitro*.

2. Malaria Drugs STEP Q, STO-AQ, STO-A4, STO-A5

Managed the implementation of departmental quality practices for the execution of studies in agreement with MRMC policies and US FDA standards in support of IV AS drug development program. Work involved the generation and/or revision of nearly 50 SOPs; upkeep of personnel training and qualification records; space utilization for LCMS lab, sample repository, and field clinical lab; establishment of a controlled sample tracking and inventory system; qualification of equipment used for regulated studies; and continued interaction with Medical Maintenance and service contractors. Helped integrate Departmental QA/QC efforts with those of the subsequently established QA units at the AFRIMS, WRAIR and MRMC. Participated in the IPT teleconferences, providing metabolism and pharmacokinetics insight. Chaired the PK SC undertaking tasks to address related concerns. Completed the implementation of a metabolism-based drug interaction study for AS using markers of enzymatic activity and rhesus monkeys; presented the data the ISSX and ASTMH meetings.

Spearheaded the development of a new joint AFRIMS Clinical Trails Center capable of performing regulated Phase I and Phase II studies primarily in support of STO-AQ. Developed and attained approval for a Phase 1 protocol to compare the pharmacodynamics of two formulations of IV artesunate (MIDRP funded AQ0031_05_AF). This study was subsequently placed on hold secondary to a US FDA ruling that it may not be necessary for the IV artesunate product development plan. Wrote protocol and built field site for a Phase II IV artesunate dose-ranging study that is anticipated to begin its in-life portion in the next year (MIDRP funded AQ0073_06_AF).

Between Sep 04 and Aug 05, following the administration of the specific markers (a total of 5), selected animals were co-administered a single dose of AS. Blood was collected at preset times and the plasma analyzed by LC/MS to assess the effect of AS on the metabolic profile of the marker. Drug-drug interactions following multiple AS doses were performed by co-administering the marker with the last dose of AS. The data were used to predict potential drug-drug interactions in humans. (AQ0012_04_WR: Drug-drug interaction study of artesunate in rhesus monkey).

Between Mar-June 05, five biguanide analogs were intramuscularly administered to healthy rhesus monkey. The plasma antimalarial activities were measured using P. falciparum-based bioassay and the parent drugs and their putative metabolites were measured by LC/MS. The pharmacokinetic/pharmacodynamic profiles of the biguanide compounds were characterized and a common unknown metabolite was identified following administration of 3 compounds. (A40005_04_AF: PK/PD screen of lead biguanides and their metabolites in rhesus monkey model).

Between Jan 05 and Feb 06, the method of AS/DHA analysis in human plasma using LC/MS was pre-validated. (AQ0047_06_AF: Maintenance of a cGLP Analytical Lab).

Published methods paper describing a simple, nonisotopic, semiautomated bioassay for the measurement of antimalarial drug levels in plasma or serum based on the quantization of histidine-rich protein II in malaria culture is presented. The assay requires only small sample volumes and was found to be highly sensitive and reproducible. The results closely paralleled those obtained with isotopic bioassays ($R = 0.988$, $P < 0.001$) and high-performance liquid chromatography-electrochemical detection ($R = 0.978$, $P < 0.001$).

Conducted a new Phase II dose ranging protocol of azithromycin/quinine and azithromycin/artesunate combinations for the treatment of uncomplicated falciparum malaria in collaboration with the Hospital of Tropical Diseases, Mahidol University. Efforts are partnered with Pfizer and the NIH.

Analyzed data from a clinical trial of tafenoquine monotherapy in adults for evaluation of radical curative ability and pharmacokinetics in *P. vivax* malaria. This study was fully successful in demonstrating that tafenoquine monotherapy can effectively eliminate both blood and liver stage parasites in a manner compatible with current therapies. Funded with NIH co-development grant with GSK, and partnered with Hospital of Tropical Diseases, Faculty of Tropical Medicine, Mahidol University.

Supported parasitology requirements for continuing efforts to develop a hepatocyte cell line to screen activity in the liver of antimalarial drugs and vaccine candidates. Collaboration with Department of Entomology. MIDRP funded.

3. Diagnostics/Rapid Diagnosis of Malaria STEP-L/STO-L

Continued development of real-time PCR method to reliably diagnose Pv and Pf malaria from human blood. Developed new primer and probe sets to detect malaria generically (all *Plasmodium* species) and specifically for *P. falciparum* and *P. vivax*. This development used DNA alignment software to compare the sequence of the 18S rRNA gene of *Plasmodium falciparum, vivax, malariae,* and *ovale* and *Homo sapiens sapiens*. For generic primer and probe sets, common sequences for *Plasmodium* were chosen that had differences with human sequence. For specific *Plasmodium* primer and probe sets, sequences unique for only *P. vivax* or *P. falciparum* were chosen. Chosen sequences were then checked via BLAST search for homology with any other sequence. Finally, primer and probe sets were designed using PrimerExpress$_{TM}$ (Applied Biosystems, Foster City California).

Continued work with leptospirosis diagnostic tests at the Sangkhlaburi febrile diseases study site. Surveyed 214 paired samples for IgM titers to leptospirosis using ELISA diagnostic tests (PanBio Inc., Baltimore, M.D.). Data published in January 2006.

Developed and assessed primer sets as a diagnostic for leptospirosis. Developed 5 primer sets and tested annealing temperatures and cycle curves for each set on four different cultured serovars of leptospirosis. Using one of the primer sets, confirmed the presence of leptospirosis in four clinical samples that were positive by culture. Also, determined that whole blood or packed cells were the best samples for use in leptospirosis diagnostics using PCR. We also ran 16 samples in two different runs from Nepal suspected of being positive for leptospirosis.

Using ELISA, conducted an assessment of two different proteins (*P. vivax* Circumsporozoite protein and Merozoite Surface Protein) to detect antibody responses that could be used as a surrogate marker of malaria exposure. We developed (antigen concentration and secondary antibody dilution) the ELISA with 40 different known negatives to clear background reactions. We then ran 587 different samples from a variety of anti-malarial drug clinical trials against both antigens to assess feasibility to detect increases in antibody response from malaria pre-exposure to post exposure. We found that PvCSP could detect antibody changes from baseline better in individuals who had several prior malaria exposures, but naive subjects had less of a response. PvMSP was a much better indicator. Also, antibody responses were most robust in subjects receiving no treatment or receiving mefloquine. The draft report and publication for this study is still pending.

Lastly, we evaluated the level of malaria recrudescence and/or re-infection in 16 subjects from a clinical drug resistance study in Bangladesh. The alleles of MSP1, MSP2, and GLURP were tested at pre- and post-drug treatment in malaria parasite DNA collected from each individual. Publication still pending.

In FY2006, we plan to establish wet assay characteristics for the *Plasmodium* real time PCR, establish a working leptospirosis real time PCR assay, support ongoing efforts to

develop a multiplex diagnostic PCR for use with GEIS fever study objectives, and build upon our malaria drug resistance surveillance efforts by development of real time PCR assays capable of assessing molecular markers associated with resistance.

Emerging Infectious Diseases (GEIS)

Epidemiology of Falciparum Malaria Drug Resistance Patterns in Asia:

Continued surveillance activities throughout Southeast Asia (Bangladesh, Myanmar, Thailand and Vietnam) for threat assessment of multi-drug resistant malaria. Assessed potential new field sites in Cambodia, eastern Bangladesh, Nepal, and northern Thailand (Chiang Dao). Parasite isolates continue to be analyzed longitudinally to assess for trends in antimalarial drug sensitivity patterns potentially signaling a diminution in the utility of the present armamentarium of malaria medications. GEIS funded, and coordinated with Public Health departments in the various countries.

Validated under field conditions a new non-isotopic method for *in vitro* drug resistance assays, which is simpler, as robust, and avoids radioisotopes. The methods has been made available free of charge to the malaria research community as a public service (see http:/malaria.farch.net). The test shows very reliable comparisons to the WHO microtest using a much simpler methodology. Funded by GEIS with support from Mahidol University and University of Vienna.

Initiated a combined in vitro-in vivo antimalarial drug efficacy trial of standard artesunate/mefloquine therapy in Trat province in southeast Thailand. This study, intended to investigate previously anecdotal reports of high failures using this regimen is of paramount importance in informing malaria drug policy for the greater southeast asia area. The study will be conducted employing directly observed therapy of all dosed antimalarial drugs and will draw inferences between IC50s measured using the above mentioned non-isotopic in vitro drug resistance assay.

Surveillance of Febrile Diseases along the Thai-Myanmar Border:

Published results of a multi-year effort to establish infectious etiologies to undifferentiated fevers along the Thai-Myanmar border in Kanchanaburi province: *Am. J. Trop. Med. Hyg.,* 74(1), 2006, pp. 108–113

Abstract: A hospital-based study was conducted along the Thai-Myanmar border to provide greater knowledge ofthe causes of febrile illness and to determine what zoonotic and vector-borne emerging infectious diseases might bepresent. A total of 613 adults were enrolled from June 1999 to March 2002. Cases were classified based on clinical findings and laboratory results. An etiologic diagnosis was made for 48% of subjects. Malaria was the most common diagnosis, accounting for 25% of subjects, with two-thirds *Plasmodium falciparum*. Serologic evidence for leptospirosis was found in 17% of subjects. Other etiologic diagnoses included rickettsial infections, dengue fever, and typhoid. The most frequent clinical diagnoses were nonspecific

febrile illness, respiratory infections, and gastroenteritis. Clinical associations were generally not predictive of etiologic diagnosis. Apparent dual diagnoses were common, particularly for malaria and leptospirosis. Findings have been used to modify treatment of unspecified febrile illness in the area.

Malaria Prevalence Study in Cambodia:

In 2005 investigators from the Department of Immunology and Medicine completed a country wide malaria prevalence survey in Cambodia. Partnered with several NGOs, the WHO and the National Malaria Center of the Cambodian Ministry of Health, AFRIMS provided key quality assurance oversight responsibilities, taught several malaria microscopy courses, and conducted a large portion of the parasite smear collections throughout 90 randomly selected clusters throughout the country. Results indicate that malaria prevalence is generally highest in clusters located in Rattanakiri, Stung Traeng, Preah Vihear and northern areas of Kampong Thom and Kratie. This is reflected in prevalence calculations by domain, which show that mean prevalence in domains 1, 2 and 3 were 6.9%, 2.8% and 0.2% respectively. Corresponding figures for prevalence by domain at mini-prevalence sites were 9.2%, 1.6% and 1.0%. The table below shows the prevalence of different species of malaria parasite by domain.

Parasite prevalence by domain from cross-sectional blood slide survey during household survey

Domain	P. falciparum	P. vivax	Pf + Pv	Other*	Total Positive	Negative
1	5.4 (128)	1.2 (31)	0.2 (4)	0.1 (4)	6.9 (167)	93.1 (2718)
2	1.3 (45)	1.4 (39)	0.04 (2)	0.03 (1)	2.8 (887)	97.2 (2723)
3	0.1 (5)	0.1 (5)	0 (0)	0.02 (1)	0.2 (11)	99.8 (2729)
Total	**1.8 (178)**	**0.8 (75)**	**0.1 (6)**	**0.04 (7)**	**2.7 (266)**	**97.3 (8159)**

*Other species = 7 (*P. malariae* = 6, mixed Pm+Pv = 1)

e. Future plans:

We plan to continue our multi-faceted emphasis on support for malaria product development in diagnostics, new drugs, and new vaccines. We anticipate heavy participation in a DoD wide effort on malaria microscopy QA procedures and have hired a dedicated expert teaching microscopist charged with the responsibility of developing a rigorous microscopy teaching and certification standard. Furthermore, we anticipate being the lead overseas lab for field-testing intravenous artesunate in phase I and II, as possibly phase III testing. We will continue efforts for tafenoquine development, especially towards an indication of radical cure for *Plasmodium vivax*. We will continue safety and immunogenicity testing of candidate malaria

vaccines in rhesus, and progress towards vivax challenge studies for eventual human testing of vivax vaccines in Thailand. Emerging infection work in Sangkhlaburi will continue with emphasis on flaviviruses, leptospirosis and typhoidal illnesses, and this study will be expanded to another targeted site in Nepal. Lastly, we anticipate an expand role in regional malaria surveillance with a combination of in vivo, in vitro and genetic methods to define expanding malaria drug resistance.

C. Department of Enteric Diseases, AFRIMS FY05 Research Efforts.

1. Title of research project: Surveillance of Antimicrobial Resistance of Enteric Pathogens in Indigenous Populations in Multiple Sites within Thailand

a. Investigator:

Dr. Ladaporn Bodhidatta

b. Objectives:

Monitor diarrhea etiology and antimicrobial resistance of enteric pathogens at multiple sites within Thailand.

c. Methods:

Hospitals and regional laboratories in several sites in Thailand to include Trang, Mae Hong Son, Ubon Ratchathani, Samutsakhon, Bangkok, and other locations agreed to participate. The study protocol was approved by both the US and Thai authorities. Several visits were made to each site by the Principal Investigator, as well as nursing and laboratory staff prior to study initiation. Additional equipment and supplies were provided to the microbiology laboratory at the each site. Onsite trainings were conducted. Stool samples were received at each participating site for initial assessment followed by confirmatory tests and additional laboratory studies to include molecular studies and antimicrobial susceptibility testing at AFRIMS in Bangkok Thailand.

d. Results:

Over 2,500 stool samples have been received and over 1,000 of bacterial isolates have been collected with Campylobacter and non typhoidal Salmonella as leading pathogens.

e. Future plans:

Continue study.

2. Title of research project: Development and Standardization of Realtime PCR Assays for Detection and Characterization of Enteric Pathogens

a. Investigators:

Orntipa Sethabutr
Rungnapha Phasuk
Sasikorn Silapong

b. Objectives:

Develop and standardize realtime PCR assays for the detection and characterization of enteric pathogens to include Shigella, Salmonella, Campylobacter, Cryptosporidia, Cyclospora, and Noroviruses.

c. Methods:

Based on literature review and best available sequence data, multiples sets of primers and probes were designed for each pathogen of interest. The sets were initially evaluated against cultured material. Selected sets of primers and probes were then tested against frozen stool samples collected and archived from multiple Department of Enteric Diseases studies. Lower limits of detection for several sets of primers and probes were determined.

d. Results:

Probes and primer sets have been developed and validated for Campylobacter, Shigella, Salmonella, Cryptosporidia, Cyclospora, and Noroviruses. Over 1,000 stool samples have been tested.

e. Future plans:

Continue study.

3. Title of research project: Characterization of Enteric Pathogens Isolated from Children in Phnom Penh

a. Investigators:

Dr. Bryan Smith
Dr. Ladaporn Bodhidatta

b. Objectives:

Determine diarrhea etiology and antimicrobial resistance of enteric pathogens from young children with diarrhea presenting to the National Pediatric Hospital in Phnom Penh, Cambodia.

c. Methods:

The study protocol was approved by both the US and Cambodian authorities. Additional equipment and supplies were provided to the microbiology laboratory at the National Pediatric Hospital in Phnom Penh. After obtaining informed consent, stool specimens were collected from diarrhea cases on presentation. An effort was made to obtain a matched asymptomatic control for each case. Initial stool examination and culture was performed at the National Pediatric Hospital. Confirmatory tests and additional studies to include molecular studies and antimicrobial susceptibility testing were conducted at AFRIMS in Bangkok.

d. Results:

Over 700 stool samples have been received and common diarrheal pathogens identified thus far including Rotavirus and Shigella.

e. Future plans:

Continue study for a total of two years.

4. Title of research project: Characterization of Campylobacter jejuni isolates

a. Investigators:

Oralak Serichantalerg
Piyarat Poothong

b. Objectives:

Identify and characterize a safe human challenge strain of *Campylobacter jejuni* for future vaccine evaluation.

c. Methods:

C. jejuni isolated from adult travelers during Cobra Gold exercises in 1998-2003 and Bumrungrad hospital in 2001-2002 were selected and serotyped for Penner heat stable and Lior heat labile serotyping systems. Standardized PFGE was performed on each *C. jejuni* isolate with 2 restriction enzymes. Characterization of representatives of each major cluster was done by PCR for virulence and Guillain-Barre syndrome (GBS) related genes. Isolates not containing genes associated with GBS related illness will be identified for further study.

d. Results:

346 *C. jejeuni* isolates were screened by PFGE analysis. 106 *C. jejuni* from each major cluster were characterized by PCR for virulence genes and GBS related genes. Partial

sequence of these genes has been completed, analyzed and compared to *C. jejuni* sequences in the GenBank. Potential candidate strains that lack of GBS related genes were identified and sent for further studies.

e. Future Plan: Continue study.

D. Department of Veterinary Medicine AFRIMS FY05 Research Accomplishments

1. Title of research project: Antimalarial Drugs Efficacy Testing in the Rhesus Monkey (*Macaca mulatta)/Plasmodium cynomolgi* Relapsing Malaria Model.

a. Investigator:

Dr. Montip Gettayacamin

b. Objectives:

1) Use the rhesus monkey/*P. cynomolgi* model to determine the effectiveness of new causal prophylactic and radical curative compounds which are being synthesized and developed by the US Army antimalarial drug development program.

2) Us the rhesus monkey/*P. cynomolgi* blood-stage malaria model to evaluate new antimalarial compounds for their blood schizonticidal activity.

c Methods:

Malaria is one of the most important parasitic diseases worldwide. Traditional treatment for malaria includes drugs used to prevent disease (prophylaxis) and to cure the infection (therapeutic). Antimalarial drug screening in the rhesus monkey model is very effective for making comparisons between drugs. It is fairly rapid, relatively inexpensive, and makes reliable predictions of how drugs will in act in man. Antimalarial drug screening in the rhesus monkey has played a key role in the development of every antimalarial drug licensed in the the US for the past 30 years. This model provides a mechanism to identify effective new drugs for the enhanced prevention and treatment of malaria infections.

d. Results:

In 4 experiments, using 78 monkeys, the antimalarial compound (tafenoquine) regimens were tested alone (at 0.2, 0.6, 2 and 6 mg/kg for 3 days or 12 mg/kg for 1 day) or in combination with 3-day chloroquine doses. Combination of the effective 3-day tafenoquine regimen with other antimalarials (mefloquine, Coartem and Malarone) were also screened for

radical curative activity at 6 mg/kg when given alone for three days. Data will be analyzed and used to select effective and safe combination therapies for malaria.

e. Future plans:

We anticipate testing at least two new compounds over the next calender year.

2. Title of research project: Care and Maintenance of Rhesus *(Macaca mulatta)* and *Cynomolgus (Macaca fascicularis)* Monkeys and Management of Breeding Colonies

a. Investigators:

Dr. Montip Gettayacamin
Mr. Srawuth Komcharoen

b. Objectives:

Maximize the production of specific pathogen-free rhesus and cynomolgus monkeys in the USAMC-AFRIMS production colony, using the best and most humane husbandry care, maintenance procedures, veterinary care, and disease surveillance and environmental enrichment procedures available.

c. Methods:

USAMC-AFRIMS maintains a breeding colony of rhesus and cynomolgus macaques using a closed colony system. Approximately 250 rhesus and 50 cynomolgus monkeys are used in the breeding program. Two types of breeding is managed: compatible male and female pairs are housed in special paired-type caging, and multiple harem groups are established and maintained in large gang cages. Harems consist of one breeding sire and 5-15 adult females. Newborn monkeys are weaned at approximately 6 months of age, and then are reared to adulthood in gang cages with other weanlings. All colony primates are tested routinely for the presence of infectious diseases that pose a threat to either the health of the colony or to personnel working with the primates. Humane use of the animals is assured by the intense oversight of the Institutioanl Animal Care and Use Committee. Veterinary and technical care is extensive and continuous.

Whenever possible, animals are re-utilized in multiple protocols in order to optimize the use of this limited and essential resource.

d. Results:

Forty-six (46) baby rhesus macaques were born in the colony in the last year.

e. Future plans:

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free nonhuman primates to support USAMC-AFRIMS research needs. Maintain and expand the colony by obtaining 20 new breeding males, increasing the number of paired housing cages, and placing breeding pairs in these new cages into additional animal rooms in the vivarium.

3. Title of research project: Care and Maintenance of Laboratory Rodents and Rabbits, Maintenance of Rodent Breeding Colonies, and Quality Assurance / Quality Surveillance Program

a. Investigators:

Dr. Montip Gettayacamin
Ms. Anchalee Tungtaeng

b. Objectives:

Maintain a breeding colony of specific pathogen-free laboratory rodents to meet the scientific research needs of the USAMC-AFRIMS, using state-of-the-art knowledge, equipment, and facilities.

c. Methods:

USAMC-AFRIMS maintains breeding colonies of laboratory rodents to meet the needs of AFRIMS research. Using state-of-the-art equipment, knowledge, and facilities, production is matched to the anticipated needs of individual research projects. Extensive and thorough recordkeeping ensures that outbred strains remain outbred, and that inbred strains remain truly inbred. An extensive quality assurance/quality surveillance program, which includes serologic assessments as well as necropsy/histopathologic analysis, ensures that the colony produces only high-quality disease-free animals. When necessary, new breeder stock is procured from a reliable vendor in the United States or Japan. Veterinary and technical care is extensive and continuous.

d. Results

Two thousand one hundred five (2,105) ICR mice (*Mus Musculus*) were produced for 5 active protocols. Quality assurance procedure monitors the health status of the animals produced in the colony and purchased from a local vendor.

e. Future plans:

These breeding colonies will continue to be maintained in order to provide a cost-effective means of supply of specific pathogen-free rodents to support USAMC-AFRIMS research needs.

4. Title of research project: A *Plasmodium berghei*-Mouse Model for Screening Antimalarial Drugs

a. Investigators:

Dr. Montip Gettayacamin
Pranee Hansukjariya
Anchalee Tungtaeng

b Objectives:

To evaluate potential antimalarial chemotherapeutic agents in the *P. berghei* ICR mouse - the modified Thompson Test model.

c. Methods:

The test system used for the determination of antimalarial activity of the compounds is a modification of the suppressive test known as the Thompson Test. Typically in this test, up to 22 groups of 8 mice are inoculated intraperitoneally (IP) with *P. berghei*-infected erythrocytes then treated with candidate drugs to determine the antimalarial activity. Infected erythrocytes are provided from donor mice. On experiment day 0, the donor mice are anesthetized then exsanguinated via cardiac puncture, the blood pooled and the level of parasitemia determined. The pooled blood is then diluted with normal mouse serum to a concentration of 1×10^6 P. *berghei*-infected erythrocytes per inoculum (0.1 ml). The groups of experimental and control mice are inoculated with this parasitized blood on day 0. On day 3, 4, and 5 mice are treated with either the candidate antimalarial drug or with vehicle alone, to serve as the negative control. The drug is administered orally (PO), subcutaneously (SC), intramuscularly (IM), and/or intraperitoneally (IP) up to three times a day, based on the individual and unique pharmacodynamics of the test compound. Each experimental group receives a different dose level, with up to 7 different dose groups per compound. A standard antimalarial drug may be tested along with the candidate drug for structure-activity determination and for quality assurance of the model. Blood films and body weights are taken on the third and sixth days post-infection, then at weekly intervals though day 30. Blood films are stained, examined by light microscopy, and the percent parasitemia determined. All mice are observed twice a day to assess their clinical signs. All mice with negative smears at 30 days are considered cured.

d. Results:

A total of 16 compounds were tested in 10 experiments.

e. Future plans:

This mouse model for screening new candidate antimalarial compounds has been used for over 30 years and is very effective for making comparisons between drugs. It is rapid, relatively inexpensive, and makes reliable predictions of how drugs will act in higher mammalian hosts, including humans. This is a core capability of the USAMC-AFRIMS Department of Veterinary Medicine and will be maintained so that many more compounds can be tested.

5. Title of research project: Characterization and Validation of *Anopheles dirus* Sporozoite-Induced Mouse Malaria Models (ICR mouse/*Plasmodium berghei* and *P. yoelli*) for Screening Exoerythrocytic Antimalarial Drugs

a. Investigators:

Dr. Montip Gettayacamin
Dr. Jetsumon Prachumsri, Ph.D.
Anchalee Tungtaeng
Dr. Robert S. Miller
Dr. Dennis Kyle

b. Objectives:

To evaluate potential causal prophylactic antimalarial agents in the P. yoelii mouse exoerythrocytic (EE) model at AFRIMS.

c. Methods:

A model involves infecting mice with sporozoites harvested from infected Anopheline dirus mosquitoes on day 0. The infected mice are dosed with test compound on day -1, prior to inoculation of sporozoites and then on day 1. There are 6-16 groups of 5 mice or up to 80 mice in each experiment. The routine test consists of 1 to 3 dosage levels of up to 5 compounds administered by one or two routes. One group will receive vehicle alone to serve as the control. Blood films, weight and clinical signs are followed to 30 days post-infection. Mice with negative smears at 30 days are considered to be protected.

d. Results:

Phase I of the study was continued. Four inoculation experiments (1-5 to 1-8) were completed. Four groups of donor mice were infected with *Plasmodium yoelli*, 17XL strain and sporozoites were successfully harvested from *Anopheles dirus* mosquitoes. Only 13 % of the

infected mice were lethally infected. Thus, this strain is not suitable for prophylactic antimalarial compound testing.

e. Future plans:

We plan to develop this model using a more lethal strain of malaria and validate it in FY06 using known effective drugs.

E. Department of Virology, AFRIMS FY05 Research Accomplishments

1. Title of research project: The Dengue Hemorrhagic Fever Project III: Continued Prospective Observational Studies of Children with Suspected Dengue

a. Investigators:

1. Principal Investigators:

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2. Associate Investigators:

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Alan L. Rothman, MD, Associate Professor, (UMMS)
Sharone Green, MD, Associate Professor, (UMMS)
Francis A. Ennis, MD, Director (UMMS)
Henry A. F. Stephens, Ph.D., Clinical Scientist and Head of Tissue Typing (University College London)

b. Objectives:

This study continues to investigate pathophysiologic mechanisms of illness resulting from dengue infections. Information gained from this study provides important insight into the methods of preventing and intervening in severe dengue disease. The project encompasses studies from 2003 to 2007.

c. Study Specific Objectives:

1. Characterize genetically and functionally the dengue virus-specific T lymphocyte response during, and after dengue virus infections (intracellular cytokine staining, HLA tetramers, T cell receptor gene usage).

2. Analyze interactions between dengue virus, virus-specific antibodies, and target cells in PBMC during acute dengue virus infections (quantify and characterize immune complexes, define the major cellular compartments in PBMC supporting dengue viral replication).

3. Determine if ultrasound or interstitial fluid albumin levels can predict early plasma leakage and shock. The ability to detect these shifts early in disease progression may help in prediction algorithms for DHF and permit early intervention with new therapies in the at-risk population.

4. Assess the utility of plasma sNS1 levels in predicting disease severity for subjects with primary or secondary infection due to any of the four dengue serotypes.

5. Analysis of the activation of innate immune responses in vivo during acute dengue virus infections (chemokine gene expression, inhibitory and activating NK receptor expression).

6. Identification of polymorphisms in immune response genes associated with disease manifestations and cellular immune responses during dengue virus infections (MHC class I and II, Fcγ receptor gene, KIR genes, NK receptors) and MHC class I chain-related (MIC) genes (ligands for lectin-like receptors),

7. Quantitation of viral burden in plasma and cell subsets of peripheral blood mononuclear cells (PBMC) for all four serotypes in primary and secondary dengue virus infections and determine if there is a correlation between viral load in these compartments and disease severity.

8. Measurement of neutralizing antibody elicited by primary infections, over an extended period of time. Few long-term studies of antibody titer following dengue infection have been performed previously. Neutralizing antibody will be measured on study day 1, 6 months, 1 year, and annually thereafter. Understanding wild type responses will help to set

realistic standards for vaccines. Mature secondary responses determined by neutralization six months or more after infection will be correlated with class II HLA type.

9. Determination of memory T-cell responses following primary and secondary dengue infections, over an extended period of time Understanding wild type responses and the durability of these responses over time will be crucial in setting standards for testing of candidate dengue vaccines.

10. Continue sequencing portions of the dengue genome from patients with mild dengue fever and those with severe DHF/DSS to test a hypothesis that severity of disease is strain related. In addition, compare the kinetics of plasma viral load and immune responses in primary and secondary infections with different DV serotypes.

11. Evaluate the accuracy of sequentially measured semi-quantitative d-dimer assay, as compared to standard clinical parameters, at predicting the clinical progression to severe clinical dengue.

d. Methods:

Children were enrolled if they were suspected of having an early DV infection (without evidence of DHF) or a fever without an identifiable source. Inclusion criteria included an oral temperature ≥ 38.5°C, fever onset not longer than 72 hours prior to the initial evaluation, weight > 6kg, flushed face, signed consent by parent or guardian. After informed consent is obtained, subjects are admitted to the hospital and a blood specimen obtained. The result of the plasma test for DV RNA by RT-PCR is available the morning of study day 2. Children who are DV RT-PCR-negative are given the opportunity to leave the study, or to continue in the study for clinical observation. Those children remaining in the hospital undergo inpatient observation until one day following defervescence (fever day +1). Clinical information is collected and recorded daily. Radiographic studies are performed as outlined in the protocol. Serial blood samples are collected and analyzed for **r**outine and dengue-specific blood and plasma tests were conducted to include, but not limited to:

1. CBC, WBC differential, AST, Albumin
2. Hemagglutination inhibition (HAI) assay for dengue
3. Antibody-capture DV IgM/IgG enzyme immunoassay (EIA)
4. RT-PCR for dengue, Plasma viremia titers
5. Dengue virus isolation in Toxorhynchites splendens and typing
6. IL-15, IL-18, MIP-1a, MIP-1b, and MCP-1, CD69, CD38, and Ki-67
7. Labeled antibodies to identify T cell subsets, NK cells and B cells
8. NS1 (soluble NS1 and anti-NS1 antibodies)
9. Complement assays

e. Results:

We enrolled 63 subjects from 14 Jun to 30 Nov 05. There were 32 positive PCR cases (DEN 1 = 8 DEN 2 =5; DEN 3 = 6; DEN 4 = 13; Negative PCR = 31). Alls subjects had ultrasound evaluation for plasma leakage. A subset of dengue positive cases had interstitial fluid sampling done. Three cases were lost to follow-up (all are non-dengue diagnoses). One case moved to north-east Thailand and is unavailable for follow-up. No serious adverse events occurred.

f. Future plans:

Long-term clinical follow-up is ongoing for prior years of enrollment and we are preparing for enrollment next year. Analysis for markers that predict disease severity (d-dimer, NS1 protein/antibody levels, immune activation markers), that indicate plasma leakage is or will occur, and that indicate immunity will be done. Statistical analysis of DHF resulting from primary versus secondary DV infections with regard to the role viral serotype, viral burden and virus-antibody complexes plays on resulting disease severity is planned. Characterization of the dengue specific T cell response with regard to the magnitude of T cell expansion during infection and the functional characteristics of these cells is also planned.

2. Title of research project: A Phase II, Prospective, Randomized, Double Blind, Placebo Controlled Field Efficacy Trial of a Candidate Hepatitis E Vaccine in Nepal WRAIR# 749, HSRRB Log# A-9117.1

a. Investigators:

1. Principal Investigators:
   M. P. Shrestha (WARUN)
   R. M. Scott (WARUN)

2. Associate Investigators:
   S. B. Bajracharya (SBH)
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   R. A. Kuschner (WRAIR)
   K. S. A. Myint (USAMC-AFRIMS)
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   S.K. Shrestha (WARUN)
   N. Thapa (SBH)
   C. Jhang

b. Objectives:

To evaluate the protective efficacy for the prevention of hepatitis E disease provided by the candidate hepatitis E vaccine administered according to a 0, 1 month schedule with a booster dose at month 6.

c. Methods:

A candidate recombinant baculovirus expressed hepatitis E virus (HEV) vaccine was found to be safe and immunogenic in 88 American and 44 Nepali volunteers. A 20μg formulation was selected for further evaluation in a randomized double blind placebo controlled efficacy trial in susceptible, active duty Royal Nepal Army volunteers. Of 5,571 consenting volunteers screened, 3,113 were susceptible to HEV. Two thousand volunteers (8 females, 1,992 males) were enrolled, receiving either placebo or 20μg of active vaccine. Volunteers were vaccinated at 0, 1, and 6 months with sera collected at months 0, 1, 3, 6, 7, 13, and 24. One tenth of the volunteers were followed on days 1, 3, 5, and 7 after each vaccination for local and general solicited adverse events (SoAE). Non-serious adverse events (NSAE) were recorded for 30 days after each vaccination and serious adverse events (SAE) were also collected throughout the study period. Sera and stool from cases meeting clinical and biochemical criteria compatible with viral hepatitis, were examined for HEV RNA by a reverse transcriptase-polymerase chain reaction, and serologically for HEV IgM and Ig, HAV IgM, HBsAg, HBcIgM and HCV IgG.

Of the 2000 enrolled subjects, 2000 received dose 1, 1890 received dose 2 and 1794 dose 3 (and 31 received dose 1 and 3). A total of 1566 subjects returned for concluding visit (Encounter# 8). Clinical phase closure was on 16 DEC 03.

d. Results:

A total of 402 serious adverse events recorded through out active study period, among which, 111 cases were certified as consistent with viral hepatitis by DSMB. Initial unblinding of these 111 cases was conducted on 23 JUN 04. Among 111 cases, 83 were certified as “Definite hepatitis E” and 24 were certified as “Not hepatitis E”.

The primary objective of the study was to determine the vaccine efficacy 14 days after the third dose of vaccine. The result of the analysis for the total 3-dose efficacy cohort is as follows:

There were 66 cases of definite hepatitis E among the volunteers receiving the placebo and 3 cases among those who received 20μg of the r-HEV vaccine. The vaccine efficacy was 96% with a narrow 95% confidence interval having a lower limit of 86%.

A secondary objective was to determine the vaccine efficiency after two doses of vaccine. The result of the analysis for the total 2-dose efficacy cohort is as follows:

There were 8 cases in the placebo group and 1 case in the vaccine group that fell between 14 days after the second dose and the third dose. There was statistically significant vaccine protection with efficacy in the total cohort receiving 2 vaccine doses of 87%. The 95% confidence interval for this estimate is wide, due to the small number of cases.

The vaccine was well tolerated, and no safety signals were identified.

e. Future plans:

Complete unblinding results will be obtained to distribute the results to the volunteers and concerned authorities in Nepal.

A manuscript has been generated and submitted to editorial board of New England Journal of Medicine for publication consideration.

Discussions to be planned for further development and testing of this vaccine.

3. Title of research project: Prospective Study of Dengue Virus Transmission and Disease in Primary Schools and Villages in Kamphaeng Phet, Thailand

a. Investigators:

1. Principal Investigators:
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   Dengue Office, Division of Vector-Borne Diseases
   Ministry of Public Health (MOPH)
   Mammen P. Mammen Jr, LTC, MD, MC (USAMC-AFRIMS)

2. Associate Investigators (by institution):

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3. Ananda Nisalak, M.D., Consultant in Arbovirology
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5. Chusak Pimgate, M.D., Head, KAVRU
6. Butsaya Thaisomboonsuk, Ph.D., Head, Arbovirology Clinical Section
7. Chonticha Klungthong, Ph.D., Head, Molecular Research Section

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1. James Jones, Ph.D., LTC, MSC, USA, Departmental Chief

Thai Ministry of Public Health (MOPH):

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Center for Infectious Disease and Vaccine Research, University of Massachusetts Medical School (UMMS):

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2. Daniel H. Libraty, M.D., Assistant Professor
3. Alan L. Rothman, M.D., Associate Professor
4. Sharone Green, M.D., Associate Professor
5. Francis A. Ennis, M.D., Director

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3. Amy C. Morrison, Ph.D., Assistant Research Entomologist

Department of Geography, San Diego State University:

1. Arthur Getis, Ph.D., Stephen and Mary Birch Chair of Geographical Studies
2. Jared R. Aldstadt, Ph.D. , Research Assistant, Department of Geography

b. Objectives:

The goal of the proposed study is to identify those factors that have the strongest influence on determining the early events in acute DV infections, and the eventual clinical manifestations of disease. An equally important goal is to characterize protective immune responses (e.g. $CD4^+$ and $CD8^+$ T-cell responses, neutralizing antibody responses) as we have found that low levels of pre-existing neutralizing antibodies to a subject's own infecting virus isolate do not necessarily protect from symptomatic DV infection. We plan to prospectively identify host-specific factors (e.g. pre-existing memory T and B cell responses to DV, HLA genetic polymorphisms, viral burden and replication in the host), virus-specific factors (e.g. DV serotype, serotype infection sequence), and environmental factors (e.g. mosquito population patterns, mosquito viral burden) for asymptomatic and symptomatic secondary DV infections, particularly severe infections (DHF/DSS). Multi-year investigations are crucial to this study due to the year-to-year variations in the incidence and prevalence of circulating serotypes. An improved understanding of the correlations between the host, viral, and environmental factors and dengue disease severity will contribute to DV vaccine development and testing.

c. Study Specific Hypotheses:

1. Subjects with pre-existing neutralizing dengue antibodies above a definable threshold will be protected from DV infection or severe disease on subsequent exposure to virus.

2. The frequency of pre-existing $CD4^+$ and $CD8^+$ T-cells and their specific cytokine responses to stimulation with DV antigens will correlate with disease severity (protection or enhancement) and the plasma viral RNA levels measured in secondary DV infections.

3. Specific serotype sequence combinations of DV infections will elicit qualitatively and quantitatively distinct immune responses associated with illness of varying severity.

4. Higher viremia levels will be seen in secondary DEN-2 and DEN-4 virus infections in subjects with higher levels of *in vitro* antibody-dependent enhancing capability of pre-illness blood samples.

5. DV infection rates will cluster in households around a DV-infected index case and a correlation will exist between the number of susceptible contacts, and associated mosquito density, and mosquito infectivity (viral RNA levels).

6. DV disease severity will correlate with peak plasma viremia levels and associated mosquito density and mosquito infectivity (viral RNA levels).

7. Genes encoded within the human MHC, the NK killer inhibitory receptor (KIR) gene complex on chromosome 19, and the Fc gamma receptor gene complex on chromosome 1 influence the susceptibility, severity and resistance to primary and secondary DV infections.

d. Methods:

In this study, we

i) Continue the successful prospective, school-based, study platform to study dengue epidemiology in primary school children in KPP province, and

ii) Conduct a village-based, cluster surveillance study.

a) This is a prospective school-based study of 2,000 children, which began in 2003 and will end in January 2009. Students in K2 to grade 6 are recruited and enrolled into the study. Baseline demographics are recorded and study numbers assigned. Each subsequent year, new

K1-Grade 5 students are newly enrolled. Students are followed until they are either disenrolled, withdrawn by their parent/guardian, graduate from Grade 6 or when the study ends. Every year, plasma (PBMCs for Dengue Season 1 only) are collected from the entire cohort at the beginning of the surveillance period (June). Plasma and PBMCs are collected from the entire cohort at the end of the surveillance period (January). The hemagglutination inhibition (HAI) assay is performed on paired sera from the beginning and end of the surveillance period to assess for flavivirus seroconversion. Plasma and PBMCs obtained at the end of the surveillance period in January serve as pre-illness samples in subjects who have a DV infection that same calendar year.

During the active surveillance period extending between June and November, those children who are absent from school (or who report ill to the teacher), will be evaluated either by a VHW or AFRIMS nurse using a questionnaire and oral temperature measurement. Any child who has a documented fever (temperature $\geq$ 38C) or reports illness with subjective fevers during the prior 7 days, is transported to the Public Health Office (PHO) where a public health nurse will do an evaluation. An acute blood specimen will be drawn. The child will be referred to the hospital at the discretion of the public health nurse. About 14 days later, an AFRIMS nurse visits the child to administer another questionnaire and to draw a convalescent blood specimen. The acute and convalenscent specimens are evaluated by the AFRIMS dengue/JE IgM/IgG ELISA and HAI. The acute specimen will be evaluated further by dengue RT-PCR (and virus isolation techniques).

b) Cases 'triggering' a cluster investigation are identified between Monday and Thursday of each week during the School-Based Component active surveillance period. Most specimens from acutely ill children arrive at the field station laboratory by 3pm each day. Upon arrival of the specimen, the database is reviewed to assess whether the child meets all index case inclusion and exclusion criteria. The field teams are notified of a possible case. The DV RT-PCR result (positive or negative) will normally be available by 11AM the following morning. No more than 30 positive and 30 negative clusters (as defined by the RT-PCR result of the index case) will be initiated in any given year. Once triggered, an Advance Team composed of a nurse and an entomological team supervisor visits the village and begins the consent form process. The exact location of all houses in each participating village has previously determined using a Global Positioning System (GPS) unit. Data points will be used to construct a digital map which will enable the team to precisely identify houses located within 100-200 meter radius (the exact radius to be pre-determined based on the prevalent average density of homes across all villages) of the index case and rapidly assess the likelihood of enrolling a minimum of 10 contacts. Once at least 10 contacts have been consented, the field teams will be dispatched to the village where the consent form process will continue. A clinical nurse will review the consent form, answer questions, address parental concerns, and obtain informed consent from the parents of susceptible contact children (ages 6 mo-15 yrs) residing within a pre-determined meter radius of the index household. Following the acquisition of parental consent, blood samples will be collected from 10-25 contacts. Those parents (and children) who are unavailable to be consented (and bled) are visited that same evening or the following morning. The clinical team will return to these homes approximately 5, 10 and 15 days after the initial visit to perform clinical assessments. The

children bled on day 0 (initial specimen) are re-bled on approximately day 15 (follow-up specimen). DV RT-PCR will be performed on all acute specimens. Dengue IgM/IgG ELISAs are performed on paired initial and follow-up specimens.

An entomological team collects mosquitoes, administers questionnaires, and performs insecticide spraying within the pre-determined meter radius of the index household. Another entomological team will collect mosquitoes but not perform insecticide spraying around the classroom and school bathroom areas of the index case.

KPSII 2005 report

NUMBER OF SUBJECTS ENROLLED/WITHDRAWN/APPROVED: At the beginning of the active surveillance period (1 June 2005), there were a total of 2,088 children enrolled in the school-based component. By the end of the surveillance period (1 December 2005), there were a total of 2,047.

Prior to the January 2006 bleed, 462 new children were enrolled so that a total of 2487 children were bled in January-February 2006 from 11 participating schools. Of the new enrollees, 200 (43 %) were in Grade K1. In March 2006, 298 grade 6 enrollees graduated from their primary schools and thus were withdrawn from the study resulting in an estimated maximum cohort size of 2189 for the start of the next season (June 2006).

Village-based study: During the active surveillance period (1 June to 30 November 2005), a total of 11 cluster investigations were performed, 4 clusters (involving 66 child contacts) based on dengue PCR positive index cases and 7 clusters (involving 104 child contacts) based on dengue PCR negative index cases. A total of 698 blood specimens were drawn from school surveillance.

SUMMARY OF RESULTS:

School component: During the active surveillance period from 1st June –30th November 2005, 764 illness cases were identified, either with fever on evaluation or reporting subjective fevers within the prior 7 days. Of these, 26 were serologically positive for dengue. 24 were Acute Secondary Dengue Infection, 1 was Acute Primary Dengue Infection and 1 suggested Recent Secondary Dengue Infection. From 26 confirmed dengue infection 20 cases were RT-PCR positive and DEN-4 was the predominant serotype (75%). Four children from the school component were hospitalized with confirmed dengue, 3 with dengue fever and 2 with dengue hemorrhagic fever (DHF). Of these, one served as an index case for a cluster investigation and all were hospitalized in August and September.

Village component: Upon day 0 evaluations, 2 of 66 enrollees within positive cluster investigations were dengue PCR positive and none of 104 enrollees within negative cluster investigations were dengue PCR positive. Upon day 15 evaluations, none of 66, 104 contacts within positive cluster and negative cluster investigation were dengue PCR positive. Within the

positive cluster investigations, there were 3 Acute Primary Dengue Virus Infections, 3 Acute Secondary Dengue Virus Infections and one Recent Secondary Dengue Virus Infection amongst contacts. All paired sera from negative cluster investigations were without dengue seroconversion by ELISA. As part of the entomological study, 3 of 425 collected Aedes mosquitoes were positive by RT-PCR (1 DEN-2, 2 DEN-3); all 3 were collected from two positive cluster investigations (#3-06 and 3-09). The dengue serotype of the mosquitoes was identical to the serotype of the index cases.

School-based active surveillance for 2005 conducted by village health workers visiting 11 primary schools during school days began in June 2005 (see Table).

1. School-based study

1.1 Number of cases related to fever and bled in 2005

School no.	T≥38°C on evaluation during a given illness	If<38°C but child reports subjective fevers during and 7 days prior	Children with reported or suspected fever (within past 7 days)	Bled (acutely and 14±4 days later)	Percent of recent fevers who were bled
01	26	42	68	61	89.71
02	32	51	83	76	91.57
03	23	9	32	31	96.88
04	23	17	40	38	95.00
05	9	12	21	17	80.95
06	23	33	56	47	83.93
07	51	85	136	127	93.38
08	38	94	132	115	87.12
09	32	34	66	66	100.00
10	18	22	40	40	100.00
11	39	51	90	80	88.89
Total	314	450	764	698	91.36

From the 698 acute bleeds, there were 26 children serologically confirmed dengue infection, from theses 24 were Acute Secondary Dengue Infection, 1 was Acute Primary Dengue Infection and 1 suggested Recent Secondary Dengue Infection.

The total numbers of dengue serotype positive with confirmed by serology were 20 febrile children. The dengue serotype predominant in these serologically confirmed was DEN-4 (15 cases or 75 %). 6 JEV infection serologically interpretation but they had no signs and symptoms of Encephalitis.

1.2 Serology with Dengue RT-PCR results

Serology	Serotype					Total
	DEN1	DEN2	DEN3	DEN4	NEG	
Acute Primary Dengue Infection					1	1
Acute Secondary Dengue Infection	2	2	1	15	4	24
JEV Infection					6	6
Need Follow Up Specimen					1	1
No Evidence of Recent Flavivirus Infection					662	662
Recent Secondary Dengue Infection					1	1
Recent Secondary Flavivirus Infection					1	1
Single specimen					2	2
Total	2	2	1	15	676	698

1.3 School - based Acute Illness RT-PCR Dengue Serotype with confirmed EIA

School No.	DEN-1	DEN-2	DEN-3	DEN-4	NEG	Total
01					60	61
02		1			75	76
03					31	31
04			1		37	38
05					17	17
06					47	47
07				13	114	127
08	2	1		2	110	115
09					65	66
10					40	40
11					80	80
Total	2	2	1	15	676	698

2. Village-based study

At the end of 2005 we had a total of 11 cluster investigations, 170 contacts, 4 positive clusters and 7 negative clusters.2 negative clusters were initially identified as positive clusters but the serology not confirmed. Enrollees in 4 positive clusters were 66 and 104 in negative ones. The serology of all positive cluster indexes were Acute Secondary Dengue Infection, dengue RT-PCR serotype were 2 indexes of DEN-1, 1 index of DEN-2 and 1 index of DEN-3.

In all 104 negative contacts there were no RT-PCR (day0, day 15) and serology positive but in the 66 positive contacts, we found 6 serology positive, 3 Acute Primary Dengue Infection and 3 Acute Secondary Dengue Infection. 2 from 3 positive Acute Secondary Dengue Infections were positive for DEN-1 and DEN-3 on day0.

Results of 425 Mosquitoes collecting were shown 1 Ae. aegypti positive DEN-2 in DEN-2 index cluster cluster3-06 and 2 ones positive DEN-3 in DEN-3 index cluster3-09.

2.1 Cluster investigation

Cluster no.	Subject no. of Index case	School no.	Type of cluster	Serotype	Serology	Number of Enrollees		Number of Houses
						Female	Male	
3-01*	030443KDS31	03	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	11	14	31
3-02	030446KDS31	03	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	6	8	27
3-03*	061108KDS31	06	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	6	4	13
3-04	040848KDS31	04	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	5	7	17
3-05	081550KDS31	08	Positive Cluster	DEN1	Acute Secondary Dengue Infection	10	15	37
3-06	020147KDS31	02	Positive Cluster	DEN2	Acute Secondary Dengue Infection	5	6	20
3-07	020210KDS31	02	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	6	6	15
3-08	081520KDS31	08	Positive Cluster	DEN1	Acute Secondary Dengue Infection	8	4	18
3-09	040877KDS31	08	Positive Cluster	DEN3	Acute Secondary Dengue Infection	8	10	15
3-10	101816KDS31	10	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	5	5	16
3-11	081532KDS31	08	Negative Cluster	NEG	No Evidence of Recent Flavivirus Infection	12	9	42

*initially positive cluster by RT-PCR but serology negative

2.2 Positive clusters

Cluster No.	Index PCR (day -1)	No. contacts	No. Contact with serologically confirmed dengue			No. with day 0 ⊕ PCR				No. with day 15 ⊕ PCR				No. Mosq ⊕ PCR (Female Ae.aegypti)			
			1°	2°	Other*	DEN-1	DEN-2	DEN-3	DEN-4	DEN-1	DEN-2	DEN-3	DEN-4	DEN-1	DEN-2	DEN-3	DEN-4
3-05	DEN-1	25	1	1		1											
3-06	DEN-2	11	1		1										1		
3-08	DEN-1	12	1														
3-09	DEN-3	18		2				1								2	
Total		**66**	**3**	**3**	**1**	**1**		**1**							**1**	**2**	

*** other Recent Secondary Flavivirus Infection, propable JE**

f. Future plans:

The results of 2004 and 2005 seasons was presented at a symposium of the American Society of Tropical Medicine and Hygiene meeting in December 2005 in Washington, D.C.

In 2006, we plan to complete analysis of 2004-2005 transmission seasons and plan to continue cluster investigations. A manuscript is in preparation.

4. Project Title: A Phase I/II Trial of a Tetravalent Live Attenuated Dengue Vaccine in Flavivirus Antibody Naive Infants

a. Background:

The US Army seeks to acquire a licensed vaccine capable of protecting soldiers and their families from disease caused by infection with the dengue viruses. The Kingdom of Thailand shares this goal. For over 50 years the US Army has been active in developing and testing various vaccine candidates. This study represents the first use of the most promising Army dengue vaccine candidate in an overseas (Thailand), infant population.

b. Objectives:

1. To demonstrate the WRAIR tetravalent dengue vaccine is safe and well-tolerated in a small number of Thai infants between the ages of 12 and 15 months.

2. To assess the immunogenicity of the dengue vaccine in terms of seroconversion 30 days post-dose 2 of dengue vaccine

c. Methods:

1. Screen and enroll 51 healthy, flavivirus naïve, Thai infants between the ages of 12 and 15 months.

2. Provide 2 doses of the WRAIR tetravalent dengue vaccine as outlined in the study protocol.

3. Closely monitor the infants following each dose of vaccine for safety and tolerability.

4. Assess the immunogenicity of the dengue vaccine as outlined in the study protocol.

d. Principal Investigators:

Mammen P. Mammen, Jr. LTC, MC, USAMC-AFRIMS
Sriluck Simasathien, MD, Phramongkutklao Hospital (PMK), Bangkok, Thailand

Additional Study Personnel:

Rudiwilai Samakoses, MD (PMK Hospital)
Angkool Kerdpanich, MD (PMK Hospital)

Veerachai Watanaveeradej, MD (PMK Hospital)
Ananda Nisalak, MD (AFRIMS)
Bruce L. Innis, MD (GSK)
Wellington Sun, MD (WRAIR)
Robert V. Gibbons, MD (USAMC-AFRIMS)
Stephen J. Thomas, MAJ, MC (WRAIR)
Kenneth H. Eckels, PhD (WRAIR)
J. Robert Putnak, PhD (WRAIR)
Celia Barberousse (GSK)

e. Results:

Following the initial testing in U.S. adults and Thai children, the Walter Reed Army Institute of Research (WRAIR) live attenuated tetravalent dengue vaccine (Formulation 17) is currently being tested in this study in a randomized, observer-blind, controlled fashion to assess for the safety and immunogenicity of the vaccine in Thai infants. The infants received 2 doses of dengue/control vaccine six months apart followed by two doses of Japanese encephalitis (JE) vaccine. Fifty-one flavivirus-naïve (lack of prior exposure to dengue virus nor JE virus/vaccine) infants (12 – 15 months of age) have been randomized to receive either dengue vaccine or control vaccine (Varicella vaccine for dose 1 and Hemophilius B vaccine for dose 2). Fifty-one healthy male and female, flavivirus naïve infants were enrolled. Ninety-six (96) infants were screened for enrollment. Fifty-one (51) study subjects were selected and assigned to one of three cohorts (cohorts A, B and C). Fifty infants (98%) completed all study visits. According to Protocol Analysis (ATP) involved forty- nine infants. One subject dropped out (migration from study area) (subject 47, control group). Another subject (subject 18, dengue group) experienced a wild-type dengue virus infection during the study and was eliminated from the ATP immunogenicity cohort. The clinical follow-up visits of Dengue-001 amendment 4 have been completed. All infants tolerated the vaccinations without SAEs attributed to vaccination. Dengue vaccine at 1/10 dose did not provide a more advantageous safety profile. The longer-term monitoring of these infants (Dengue-001 amendment 5) was approved by all IRBs .

Screened	Cohort Enrollees	Dengue/ Control	Remarks
14	A (6 infants)	4 /2	1/10 of full dose (6 infants completed doses 1 and 2 of Dengue/Control vaccine)
28	B (15 infants)	10/5	Full dose (15 infants completed doses 1 and 2 of Dengue/Control vaccine)
54	C (30 infants)	20/10	Full dose (30 infants completed dose 1 of Dengue/Control vaccine) 1 infant was withdrawn from the study

f. Future plans:

The results of the study were presented at the World Health Organization (WHO) Flavivirus Steering Committee meeting in Washington D.C. in December 2005.

Based on favorable safety and immunogenicity data, this vaccine will be further studied in Thailand in adults and pediatric trials by AFRIMS in cooperation with the Thai MOPH and GSK.

5. Title of research project: Training and Workshops

a. Background:

The Department of Virology, Armed Forces Research Institute of the Medical Sciences (AFRIMS), Bangkok, Thailand, seeks to expand its diagnostic capabilities in South and Southeast Asia by improving regional laboratory capabilities through the dissemination of diagnostic kits and the training of technical personnel.

b. Goals:

1. To create and improve the laboratory infrastructure of South and Southeast Asian regional laboratories specializing in infectious disease surveillance.

2. To provide the training of laboratory personnel (technicians and supervisors) working in South and Southeast Asia and beyond in infectious disease diagnostic techniques.

c. Activities:

The department conducted numerous on-site and in-house diagnostic training activities.

1. Over 50 student scientists from Srinakarinviroj University, Chulalongkorn University, Phramongkutklao Medical College, Faculty of Tropical Medicine-Mahidol University received 2 to 4 weeks of training at AFRIMS in diagnostic laboratory modalities.

2. On-site training (Kathmandu, Nepal) in the proper performance of the AFRIMS JE EIA and basic instruction in QA and QC principles was provided to representatives of numerous Nepali health institutions.

3. Training in the proper performance of the AFRIMS JE EIA and basic QA and QC principles was provided to visiting scientists from the National Institute of Hygiene and Epidemiology, Hanoi, Vietnam, and National Institute of Pediatrics, Hanoi, Vietnam.

4. During the past year diagnostic kits or training was provided to the following laboratories:

B.P. Koirala Institute of Health Sciences, Dharan, Nepal
Nepal Public Health Laboratory, Kathmandu, Nepal
Teku Hospital, Kathmandu, Nepal
Bheri Zonal Hospital, Nepalgunj, Nepal
Institute of Medicine, Kathmandu, Nepal
ICDDRB, Dhaka, Bangladesh
Pasteur Institute, Ho Chi Minh City, Vietnam
Research Institute of Tropical Medicine, Alabang, Philippines
San Lazaro Hospital, Manila, Philippines

6. Title of research project: Influenza Surveillance in Southeast Asia

a. Background:

Influenza is an important cause of morbidity and mortality among populations at the extremes of age. Continuous viral surveillance and isolation of influenza viruses provides important information for the creation of annual vaccine formulations based on the identification of new and emerging strains of influenza. AFRIMS has been actively involved in influenza surveillance in Thailand and Nepal for several years. Expansion of AFRIMS influenza surveillance activities in the region will enhance DoD's ability to detect and respond to an outbreak of pandemic influenza early in the course of the pandemic.

b. Goals:

(a) To collect and characterize influenza viruses circulating within the human population in Asia including Thailand, Nepal and the Philippines.

(b) To provide influenza surveillance data to the US CDC and WHO surveillance network towards the annual re-formulation of the influenza vaccine.

(c) To report the circulating influenza strains and other respiratory pathogens to the Ministry of Health of host countries.

c. Activities:

Samples were collected from patients with clinically suspected influenza infection (case definition includes fever or history of fever $\geq$38°C within 72 hours with cough or sore throat). Participating physicians and staff identified patients who met the case definition during routine clinic visits. Emphasis was placed on quality samples that may provide genetic data for future influenza vaccines rather than a large number of samples to be tested for incidence and prevalence data. Clinical history forms, including basic demographic and clinical information, were completed by the OPD nurse or AFRIMS research nurses. Throat swabs were collected and placed in viral media and stored at -70°C. All specimens were shipped on dry ice to AFRIMS,

which in turn shipped the samples to Armstrong Laboratory, Brooks AFB. Rapid diagnostics for Influenza (QuickVue) were field tested at the sites in Sankhlaburi, Kamphaeng Phet in Thailand, the medical facilities of the US Embassy in Bangkok, Thailand, and Kathmandu, Nepal.

d. Progress:

AFRIMS continues to work in close collaboration with the US and Thai CDCs, the Thai Ministry of Public Health, and with NAMRU-2. The influenza surveillance is divided into individual country projects each for Thailand, Nepal, US Embassies in the region and the Philippines. Provision of staff, equipment, infrastructure development, and training is underway. Floor plans to establish a dedicated respiratory pathogens laboratory has been submitted. This will allow immediate processing of influenza samples, and ensure on-time reporting. The laboratory will be equipped with a real-time pcr machine, serology set up, viral isolation, computers for data entry, and capabilities for specimen storage and archiving.

Sequence data from influenza isolates obtained by AFRIMS from outbreaks in Nepal in 2004 and 2005 were used to modify the composition of the annual influenza vaccines. 118 specimens from the US Embassy Bangkok have been collected and tested since September 2005.

e. Future plans:

1. To expand a site to include Manila (San Lazaro Hospital) in the Philippines.
2. To expand surveillance to other countries in the region (Bhutan, Maldives)

f. Investigators:

1. Khin Saw Myint, M.D.
2. Robert V. Gibbons, M.D. MPH
3. Mammen P. Mammen, Jr. M.D.
4. Chusak Pimgate, M.D.
5. Charity Ann Ypil-Butac, M.D.
6. John Mark Velasco, M.D.
7. Rodney Coldren, M.D.

7. Project Title: Sentinel Surveillance for Emerging Diseases Causing Dengue-like or Acute Encephalitis Syndrome in the Philippines (SEDP)

a. Principal Investigators:

Armed Forces Research Institute of Medical Science (AFRIMS):
Department of Virology

Mammen P. Mammen, Jr. LTC, MC (USAMC-AFRIMS)
Maria Theresa Alera, M.D (San Lazaro Hospital, Manila, Philippines)

b. Other Study Personnel:

Armed Forces Research Institute of Medical Science (AFRIMS):
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1. Charity Ann Ypil-Butac, M.D.
2. John Mark Velasco, M.D.
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3. Richard G. Jarman, PhD
4. Ananda Nisalak, M.D
5. Butsaya Thaisomboonsuk, PhD
6. Piyawan Chinnawirotpisan, PhD
7. Thidarat Intararit, R.N.

Department of Entomology

1. James Jones, Ph.D., LTC, MSC

San Lazaro Hospital

1. Efren Dimaano, M.D

Philippine-Department of Health (DOH):

1. Lyndon Leesuy, M.D.
2. Vito G. Roque, Jr., M.D.
3. Mario S. Baquilod,M.D.,MPH

World Health Organization (WHO)

1. Raman Velayudhan, M.D.

University of the Philippines – Entomology Department

1. Lillian A. de las Llagas, PhD

c. Objectives:

Primary objectives: Epidemiology

General:

To determine the spectrum of emerging diseases causing dengue-like syndrome (DLS) or acute encephalitis syndrome (AES) in the Philippines

Specific:

(1) To determine the proportion of dengue-like syndrome that is caused by:

a. Dengue

b. Leptospirosis
c. Chikungunya
d. Scrub typhus
e. Murine typhus

(2) To determine the proportion of acute encephalitis syndrome that is caused by:

a. Japanese encephalitis
b. Rabies
c. West Nile

Secondary objectives: Laboratory and Public Health Infrastructure

1. To enhance the diagnostic capabilities of the Philippines.

2. To determine the mosquito species associated with the transmission of Japanese encephalitis (JE) and West Nile viruses.

d. Methods:

Study Design:

This was a cross-sectional, hospital-based passive surveillance study conducted among admitted patients in San Lazaro Hospital, a tertiary government hospital located in Manila, Philippines

Study Population:

The study population consisted of male and female patients 2 years old and above who present with dengue-like syndrome or acute encephalitis syndrome and who signed an informed consent to participate in the study. Patients with DLS were enrolled in the study if they met the following inclusion criteria: any patient with history of fever (temperature of 38^{o}C and above) within the past 2-7 days with either one of the following criteria: a positive tourniquet test, an eschar, migratory polyarthritis or calf pain ***OR*** two of the following, namely: headache, generalized rash, myalgias, arthralgias, retro-orbital pain or icteric sclerae. Patients with AES who present with acute onset of fever and a change in mental status (including symptoms such as confusion, disorientation, coma, or inability to talk) and/or new onset of seizures (excluding simple febrile seizures) were also enrolled. Criteria for exclusion were explainable causes of DLS or AES, AES preceded or associated with exanthem and known pregnancy.

Data Collection:

Patients who met the enrollment criteria were interviewed and examined. The patient's personal, demographic, clinical and laboratory data were recorded. Acute blood specimens were drawn. In addition, CSF samples were obtained from AES patients.

Initial laboratory testing focused on probable etiologies given the recognized endemic pathogens. Cases that remain undiagnosed after the initial stage of testing were further characterized using specialized testing to identify emerging pathogens. Patients were advised to come back 14 days after onset of illness where a convalescent serum was drawn.

Laboratory testing was conducted in Stages:

Stage 1: laboratory tests routinely ordered by the San Lazaro Hospital clinical team as part of the management of the cases based on the prevailing standards of care of the various conditions in the Philippine setting

Stage 2: AFRIMS in-house DEN/JE Enzyme immunoassay (EIA) was performed to help rule in/rule out either dengue or JE etiology; specimen with negative/inconclusive results were subjected to the next level of testing

Stage 3: laboratory tests performed for presumptive diagnosis

Stage 4: confirmatory laboratory tests and other more advanced tests

Final diagnosis was based on on study-specific case definitions. Cases which yielded negative results after Stage 4 testing were classified as those belonging to other etiologies.

The collaborating institutions were provided with laboratory test capabilities to perform AFRIMS in-house DEN/JE EIA. The investigators ensured that the materials required for the test performance were provided. On-site training of laboratory personnel and quality monitoring was conducted by AFRIMS staff. Proficiency evaluation was done.

e. Results:

DLS Component

A total of 275 patients were screened and 104 patients were enrolled for the DLS component of the study. Forty (38%) were female and 64 (62%) were male. Most of the patients - 89 (86%) came from the National Capital Region (NCR) while the rest came from other areas in Luzon. Out of the 104 patients enrolled for DLS, 90 patients were confirmed to be positive for dengue with 87 patients showing ELISA seroconversion on acute and convalescent sera, 1 patient with ELISA-positive acute specimen and 2 with PCR- positive acute serum.The following diagnosis were noted among the 14 patients who were considered as non-dengue cases:1 with Leptospirosis, 1 with Disseminated PTB (mortality), 1 with Malaria P. falciparum infection, 1 with Pneumonia, 1 with Typhoid Septicemia (mortality) and 1 with Chikungunya. Results from other specialized assays are still pending.

Though dengue is considered a pediatric disease, results from this study showed an equal distribution of the pediatric and adult cases. Patients belonging to the pediatric and adult

age groups were noted to be clinically indistinguishable. Technology transfer of dengue diagnostic capability to San Lazaro Hospital resulted in high concordance for both Dengue IgM and IgG EIA. The EIA results showed that most of the patients had acute secondary dengue infection. Though all serotypes were identified during the study period, DEN-3 was noted to be the predominant serotype. There were 2 mortalities among DLS cases. Seven patients did not come back for follow-up.

AES Component

Clinical diagnosis of the 8 patients who were enrolled for the AES component are as follows: 5 with Viral encephalitis, 2 Viral encephalitis with Pneumonia, 1 Viral encephalitis with Pulmonary Tuberculosis (PTB). One patient was noted to have acute secondary dengue on EIA with nested PCR showing DEN-3 infection. Additional laboratory testing is ongoing. One AES patient died while the rest were noted to have neurologic sequelae upon discharge.

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f. Future plans:

Future plans include extension of the DLS component to a tertiary government hospital in Cebu City,Philippines and extension of AES component to the encephalitis belt (Tarlac and Bulacan provinces) in northern Luzon, Philippines.

F. Department of Retrovirology, AFRIMS FY05 Research Accomplishments

1. Title of research project: Screening and Evaluation of Potential Volunteers for a Preventive HIV-1 Vaccine Trial in Thailand (RV148, HSRRB)

a. Investigators:

Dr. Supachai Rerks-Ngarm, Dept. of Disease Control, MOPH;
COL Sorachai Nitayaphan, RTA Component, AFRIMS;
Dr. Jaranit Kaewkungwal, Mahidol University.

b. Objectives:

To evaluate adult Thai volunteers for eligibility and subsequent enrollment in a preventive HIV-1 vaccine trial (Phase III) of a prime-boost vaccine combination for the prevention of HIV infection.

c. Methods:

Volunteers who indicate an interest in participating in HIV vaccine research will receive information and education about the upcoming vaccine trial. They will be evaluated to see that they meet the eligibility criteria. They will receive counseling and education on HIV,

aspects of participating in a HIV vaccine trial, and will be tested for HIV-1 infection by a standard ELISA & Western blot algorithm. Those volunteers who test positive for HIV will have CD4 enumeration and HIV viral load testing. Those that are eligible for the Phase III study and pass a test of understanding will be offered enrollment in a vaccine efficacy trial.

d. Results:

The protocol began enrolling volunteers as of 29 September 2003. Over 26,000 persons were screened. Screening ceased 30 December 2005.

e. Future Plans:

This protocol is closed.

2. Title of research project: A Phase III Trial of Aventis Pasteur Live Recombinant ALVAC-HIV (vCP1521) Priming with VaxGen gp120 B/E (AIDSVAX® B/E) Boosting in HIV-uninfected Thai Adults (RV144, HSRRB Log No. A-11048, BB-IND 8795).

a. Investigators:

Dr. Supachai Rerks-Ngarm, Dr. Supamit Chunsutthiwat- Department of Disease Control, Ministry of Public Health, Nonthaburi, Thailand
COL Sorachai Nitayaphan, RTA Component, AFRIMS
Prof. Punnee Pitisuttithum, Mahidol University
Assoc. Prof. Jaranit Kaewkungwal, Mahidol University

b. Objectives:

Primary: To determine whether immunizations with an integrated combination of ALVAC-HIV (vCP1521) boosted by AIDSVAX® gp120 B/E prevent HIV infection in healthy Thai volunteers. Secondary: To determine whether immunization with this vaccine combination results in reduced HIV viral load "set point" among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To determine whether immunization with this vaccine combination results in an increased CD4 count measured at viral load "set point" among those acquiring HIV-1 infection, comparing vaccine recipients to placebo recipients. To confirm the safety of this vaccine combination in Thai volunteers.To evaluate whether participation in this HIV vaccine trial is associated with behavior change that may increase the risk of HIV infection.

c. Methods:

This will be a community-based, randomized, multicenter, double-blind, placebo-controlled clinical trial (vaccine:placebo = 1:1). Screening of potential volunteers will be carried out under a separate protocol entitled "Screening and evaluation of potential volunteers for a trial in Thailand of a candidate preventive HIV vaccine" (RV148). Eligible volunteers will be

enrolled over approximately one year. The statistical assumptions of the study will require that 16,000 persons enroll into the study. Vaccinations for each individual will occur over a 24-week period (0, 4, 12, 24 weeks). Women will be tested for pregnancy and pregnant volunteers will not be vaccinated. The volunteers will be followed with HIV testing every 6 months for 3 years after immunization. Blood will be collected for plasma (for diagnostics and HIV-specific antibodies) at 0, 24 and 26 weeks, and every 6 months during the follow-up phase. The blood collection at 0 and 52 weeks will also be used for cryopreservation and archiving of PBMCs (for HIV-specific cellular immune responses). At week 24 and at each six-month follow-up visit, volunteers will have HIV testing, preceded by pretest counseling and followed (approximately 2-3 weeks later) by post-test counseling. Assessment of HIV risk behavior will be performed at baseline and at each 6-month follow-up visit. Education on risk behavior reduction will be given at each vaccination visit and at each post-test counseling visit.

d. Results:

The first volunteer injected on 20 October 2003. As of December 2005, 16,402 volunteers were enrolled and enrollment has ceased.

e. Future Plans:

All vaccinations will be performed by 31 July 2006. Volunteers will be followed for 3 years after vaccination. A Data and Safety Monitoring Board meeting was held 4 – 5 October 2005 and no safety concerns were raised. A Data and Safety Monitoring Board meeting is being held 25- 26 June 2006.

III. APPENDICES:

A. PERSONNEL ASSIGNED UNDER AGREEMENT

Department of Administration

1. Ms. Bang-on Kesdee
2. Mr. Weerasak Yeephu
3. Mr. Sompol Boonnak
4. Ms. Pattrapan Jullasing
5. Ms. Geerati Sornwattana
6. Ms. Yinglak Apisitsaowapa
7. Ms. Tippawan Tephassadin na Ayuthaya
8. Mrs. Somporn Krasaesub
9. Mr. Theerasak Ponepan
10. Mr. Prinya Yoophasook
11. Mrs. Khanitha Rojanasthien
12. Mrs. Bussara Sukpanichnant
13. Mrs. Lakhana Phaoharuhansa
14. Mr. Danuphol Junkaew
15. Ms. Usa Panichpathompong
16. Mrs. Sirin Limsurat

Department of Logistics

17. Mr. Sawadi Boonnak
18. Mr. Charan Kajeechitr
19. Mr. Thongchai Duangkeaw
20. Mr. Boonthum Jamjank
21. Mr. Komson Boonnak
22. Mr. Somporn Pinpo
23. Mr. Chatchai Sang-ngen
24. Mr. Prasitchai Kruaysawat
25. Mr.Yuthana Seemat
26. Mr. Siriphong Amnuaisuksiri
27. Mr. Patrabhum Kwanyou
28. Mrs. Anchisa Maleenun
29. Ms. Nongnoot Chaowaratana
30. Ms. Panisara Puangsilpa
31. Mr. Tharanat Thanatepisansakun
32. Ms. Yuwadee Sake-Yonog
33. Ms. Saruta Anukul

34. Mr. Peeradon Gabriel

Department of Logistics (continued)

35. Ms. Ruedee Arunkhajohnsak
36. Mr. Jakkapong Maneerut

Department of Immunology

37. Mrs. Barnyen Permpanich
38. Ms. Amporn Limsalakpetch
39. Ms. Nillawan Buathong
40. Mr. Chaiyawat Mathavarat
41. Mr. Prasit Sookto
42. Mrs. Somchit Tulyayon
43. Ms. Srisombat Wannaying
44. Ms. Apassorn Lim
45. Ms. Sabaithip Sriwichai
46. Ms. Duangkamon Siludjai
47. Ms. Monticha Kongthaisong
48. Ms. Suthatta Hanwisai
49. Mr. Sittidech Surasri
50. Mr. Worachet Kuntawunginn
51. Dr. Youry Se

Department of Virology

52. K.Y. Dr. Ananda Nisalak
53. Mrs. Chuanpis Ajariyakhajorn
54. Mrs. Sumitda Narupiti
55. Mrs. Naowayubol Nutkumhang
56. Mrs. Vipa Thirawuth
57. Ms. Panor Srisongkram
58. Mr. Somsak Imlarp
59. Mr. Wichien Sa-Nguansuk
60. Mr. Prachakkra Panthusiri
61. Ms. Wallika Kulthongkam
62. Mr.Wanchai Inpho
63. Mrs. Pannarat Chuakanubon
64. Mrs. Rungkarn Hangsuwan
65. Mr. Yongyuth Poolpanichupatam
66. Mr. Thanawat Boonnak
67. Ms. Rattiya Wannawong
68. Ms. Prinyada Rodpradit
69. Ms. Piyawan Chinnavirotpasan

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71. Mr. Wiangchai Watcharanirun
72. Ms. Wicha Panyalikhit
73. Mr. Winai Kaneechit
74. Ms. Chaleaw Saengchan
75. Ms. Nualanong Akaraputtiporn
76. Mrs. Choompun Manomuth
77. Mr. Supakit Wanasith

Department of Veterinary Medicine

78. Mr. Phongsak Maneerut
79. Mr. Thonglor Detkokao
80. Mr. Sawaeng Sripakdee
81. Mr. Phatcharaphon Jaikla
82. Mr. Rachata Jecksaeng
83. Mr. Dejmongkol Onchompoo
84. Mr. Manop Pooyindee
85. Ms. Choosri Sangsri
86. Mr. Surayuth Seegaewin
87. Mr. Manas Kaewsurind
88. Mr. Yongyuth Kongkaew
89. Mr. Vittavat Sankalee
90. Mr. Mana Saithasao
91. Ms. Anchalee Pothipoch
92. Mr. Bamrung Chaikwang
93. Ms. Siriwan Korpaiboonkij
94. Mr. Alongkorn Hanrujirakomjorn
95. Mr. Sornchai Jansuwan
96. Mr. Amnaj Andang
97. Mr. Suvit Boonkali
98. Mr. Sarayuth Chienrum
99. Mr. Sakda Sanon
100. Ms. Kwanpracha Insansueb
101. Mr. Chaisit Pornkhumviwat
102. Mr. Wuthichai Puenchompu
103. Ms. Rawiwan Im-erbsin
104. Mrs. Boonhome Meepuak
105. Mr. Amnart Kayha
106. Ms. Panida Wongprasertdee
107. Mr. Siriphong Prompim

108. Mr. Perapol Thanunok

Department of Entomology

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111. Ms. Nongnuch Yimamnuaychok
112. Mr. Yossasin Kertmanee
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114. Ms. Kanchana Pantuwatana
115. Ms. Bousaraporn Tippayachai
116. Mr. Sommai Promsathaporn
117. Ms. Sasathorn Nongngork
118. Mr. Opas Thachin
119. Ms. Warisa Leepitakrat
120. Mr. Chalermpon Kumpitak
121. Dr. Rampa Rattanrithikul
122. Ms. Jaruwan Tawong
123. Ms. Rachaneeporn Jenwithisuk
124. Mr. Somporn Chanaimongkol
125. Mr. Weeraphan Kongtak
126. MG.Vichai Sangkasuwan
127. Ms. Koraket Laptaveechoke
128. Ms. Sucheera Insuan
129. Mr. Weerayut Chareonsongsermkit
130. Ms. Nittaya Khlaimanee
131. Mr. Narong Ponsa
132. Ms. Nongnuj Maneechai
133. Mr.Udom Kijchalao
134. Mrs. Suda Ratanawong
135. Mrs. Prachong Panthusiri
136. Ms. Nantaporn Monkanna
137. Ms. Namtip Trongnipatt
138. Mr. Vichit Phunkitcha
139. Mrs. Somprathana Silapee
140. Ms. Wilasinee Akkagraisee
141. Ms. Kwanta Chayapumh
142. Mr. Pradith Mahapibul

Department of Enteric Diseases

143. Mr. Songmuang Piyaphong
144. Ms. Duangjai Lumson
145. Ms. Ovath Thonglee
146. Ms. Sasikorn Silapong

147. Ms. Rungnapha Phasuk

Department of Enteric Diseases (Continued)

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149. Ms. Ajchara Aksomboon Wongsawan
150. Mr. Boonchai Wongstitwilairoong
151. Ms. Nopparat Sae-Ier
152. Ms. Nattaya Ruamsap
153. Ms. Umaporn Suksawad
154. Ms. Piyarat Phuthong
155. Ms. Kaewkanya Nakjarung
156. Ms. Piyanate Sunyakumthorn
157. Mrs. Prani Ratarasarn
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159. Ms. Paksathorn Puripunyakom

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164. Ms. Kanya Pumratana
165. Mrs. Sujitra Santatiwongchai
166. Mr. Thiti In-ngam
167. LTG Pricha singharaj
168. Ms. Suchada Chinaworapong
169. Ms. Puangmalee Buapunth
170. Mr. Attapon Triampon
171. Ms. Pirinat Chalermpark
172. Ms. Sopana Chatnilbandhu
173. Ms. JSuchada Sukhumvittaya
174. Ms. Sakuna Suksawad
175. Ms. Nongluck Sangnoi
176. Ms. Thunyasuta Prasit
177. Ms. Nampueng Sirijongdee
178. Ms. Metta Thongtaluang
179. Mr. Siriwat Akapirat
180. Mr. Sunboon Kaewpraphan
181. Ms. Ajchariyarat Sangdara
182. Mr. Sumonsak Thojun
183. Mr. Surat Jongrakthaitae
184. Ms. Theeraporn Thamjamras
185. Mr. Nitit Isravudhakul

186. Ms. Yuwadee Phuang-ngern

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188. Ms. Marinna Nidhinandana
189. Mr. Daenangkhan Rachasaeng

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191. Ms. Siriporn Janariyawong
192. Ms. Yok Rattanathan
193. Ms. Whralak Papijit

GEIS

194. Ms. Nucharee Thongsen
195. Ms. Wonlana Theerqapolumpun
196. Mr. Supin PankoteMs. Nongluck Sangnoi
197. Mr. Chorn Thepsanan
198. Mr. Prasert Meesuksabye
199. Ms. Pattranit Soonthorntanaset
200. Mrs. Thanintorn Adeedto
201. Mr. Papungkorn Phaophuek
202. Ms. Rapida Padmasankha

****

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